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Microbial Degradation of Selected Hazardous Materials: Pentachlorophenol, Hexachlorocyclopentadiene, and Methyl Parathion

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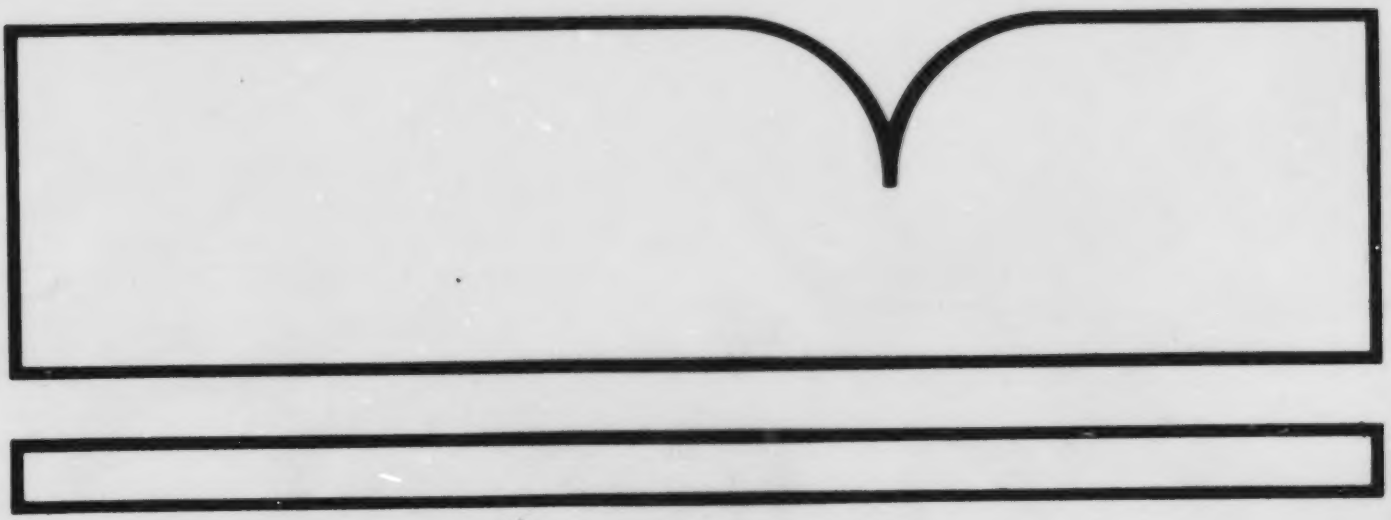

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MICROBIAL DEGRADATION OF SELECTED HAZARDOUS MATERIALS: Pentachlorophenol, Hexachlorocyclopendadiene, and Methyl Parathion

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16. ABSTRACT

This program evaluated the use of selected pure culture microorganisms for potential in biodegrading the hazardous materials pentachlorophenol (PCP), hexachlorocyclopentadiene (HCCP), and methyl parathion (MP). Each chemical was separately challenged by each of 24 organisms in aqueous medium under aerobic conditions. Following the initial screening and selection process, pure culture organisms identified as having potential for biodegradation of the selected chemicals were subjected to further testing and evaluation. Although no fully conclusive evidence of biodegradation of these substances was obtained, data indicated that a number of fungi have potential for disposal of PCP, HCCP, and MP. One bacterial culture demonstrated tolerance to PCP at 200 ppm in soil and appeared to reduce the PCP concentration in an aqueous medium when dextrose was provided. This isolate may have potential for removal of PCP from spill-contaminated areas. A fungus also showed some potential for degrading PCP.

The scope of this project did not allow for adaptation of cultures over time for biological removal of the selected chemicals. Time constraints and budgetary requirements precluded the use of C-14-labeled chemicals and the extensive analyses required for isolation, identification, and quantification of potential by-products and metabolities of the biodegradation or biotransformation of the selected chemicals. Improvements on existing HPLC and GC-EC methods were developed. However, limited data showed that, where biodegradation was presumed to have occurred, no chlorinated by-products were detected within the limits of detectability of the methods used.

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a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Biodegration Toxic Hazards Pesticides Fungicides Bacteriacides Hazardous material spills HPLC GC-EC	Fungi Bacteria Microorganisms PCP (Pentachlorophenol) HEX (HCCP, hexachlorocyclopentadiene) MP (methyl parathion) Bacteriacides	6M

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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimonies to the deterioration of our natural environment. The complexity of that environment and the interplay of its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution; it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems to prevent, treat, and manage wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, to preserve and treat public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research and provides a most vital communications link between the researcher and the user community.

This report evaluates the use of selected pure culture microorganisms for biodegrading selected hazardous materials. The work was centered on hazardous materials that have been involved in serious spills during the past few years. Test results indicate that *in situ* treatment of hazardous spilled materials with selected pure culture organisms may have potential for effective spill cleanup. For further information, contact the Oil and Hazardous Materials Spills Branch, MERL-Ci, U.S. Environmental Protection Agency, Edison, N.J.

Francis T. Mayo
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ABSTRACT

This study evaluated the use of selected pure microbial cultures for degradation of specific hazardous materials. Three compounds were selected for study: pentachlorophenol (PCP), hexachlorocyclopentadiene (HCCP), and methyl parathion (MP). Removal of these compounds from aqueous media by pure cultures of fungi, yeasts, actinomycetes, and bacteria under aerobic conditions was evaluated. A few experiments were performed to determine the effectiveness of a bacterial isolate to degrade a PCP/oil mixture in soil.

Screening tests were conducted with each chemical dispersed in an aqueous medium. Each chemical was challenged by each of 24 organisms to identify the species with potential for assimilating or metabolizing the selected substance. Organisms identified in the screening studies were used for additional tests in which treatment techniques were varied to maximize the rate and extent of disappearance of the chemical.

Although no conclusive evidence for degradation of the compounds was obtained, data suggested that some of the pure cultures have potential for the biodegradation of PCP and MP. A bacterial isolate, designated as 041, reduced the PCP concentration in the medium by 22 to 34% when dextrose was provided as a carbon source. Two fungal isolates designated as 003 and 021, appeared to reduce the MP concentration when dextrose was present in the medium. There is an unresolved ambiguity in this data on the disappearance of HCCP.

Removal of PCP by microorganisms in a four-stage pilot-plant reactor was also studied. The data suggest that two organisms, fungus 044 and bacterium 041, have potential for reducing the PCP concentration.

In tests with PCP and soil, designed to simulate the PCP-spill conditions at Haverford, Pennsylvania, it was determined that the bacterial isolate 041 tolerated higher concentrations of pentachlorophenol than the fungal culture 044. Bacterial isolate 041 may be a suitable candidate for adaptation and mutation for in-situ work to clean up such hazardous spills.

Analytical methods were based on protocols that have been published in the open literature; modifications were made as required. High performance liquid chromatography and gas chromatography (electron capture) were the most frequently used methods. Standard microbiological procedures were followed for culturing, preparing media, inoculating, and assaying. Time and funding constraints did not permit identification and quantification of metabolites and byproducts--analyses were performed only for the hazardous material being tested. In determining the percent degradation, results were corrected for material that was merely bioaccumulated.

This report was submitted in fulfillment of Contract No. 68-03-2491 by Atlantic Research Corporation under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period February 11, 1977 to May 11, 1978. Work was completed as of June 20, 1978.

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ABBREVIATIONS AND SYMBOLS

ARC #50	--ARC #50 medium
BOD	--biochemical oxygen demand (5-day)
B.S.	--basic salts
C-14	--carbon isotope with a mass number of 14 (a beta-emitter)
$CaCl_2$	--calcium chloride
cfu	--colony forming unit
CH_3CN	--acetonitrile
DO	--dissolved oxygen
EC-GC	--see GC-EC
$FeCl_3$	--ferric chloride
GC-EC	--gas chromatograph(y)-electron capture detector
HCCP	--hexachlorocyclopentadiene
HCl	--hydrochloric acid
HPLC	--high performance liquid chromatograph(y)
K_2HPO_4	--dipotassium hydrogen phosphate
KH_2PO_4	--potassium dihydrogen phosphate
km	--kilometer
LC_{50}	--concentration that results in the death of 50% of the exposed population
LD_{50}	--dosage that results in the death of 50% of the test population
m	--meter
u	--micron, 10^{-6} meter
ml	--milliliter, 0.034 fluid ounce
ul	--microliter
M.B.S.	--modified basic salts
$MgSO_4$	--magnesium sulfate
MP	--methyl parathion
1N NaOH	--one (1) normal solution of sodium hydroxide in water
NaN_3	--sodium azide
Na_2SO_4	--sodium sulfate
NH_4NO_3	--ammonium nitrate
nm	--nanometer, 10^{-9} meter
OCDD	--octachlorodibenzo-p-dioxin
PCP	--pentachlorophenol or pentachlorophenate (the sodium salt of pentachlorophenol when dissolved in aqueous sodium hydroxide)
ppb	--parts per billion (generally, mg/kg)
ppm	--parts per million (generally, mg/kg)
rpm	--revolutions per minute
s.c.	--subcutaneous
SDA	--Sabouraud dextrose agar
TCDD	--2,3,7,8-tetrachlorodibenzo-p-dioxin
TLV	--threshold limit value
TL_m	--median tolerance limit
TSA	--Trypticase soy agar
UV	--ultraviolet
#50	--see ARC #50

SECTION 1

INTRODUCTION

The impact of spilled hazardous materials on the environment is a major concern. Approximately 13,000 spills of oil and hazardous materials are estimated to occur each year (EPA, 1975). In many of these incidents, no cleanup methods are employed. For example, no cleanup action was taken in 28 of the 78 reported spill incidents that occurred between December, 1975 and May, 1976 (Mercer et al., 1978). In some of these accidents, no action was possible. Often, the technology for effective cleanup simply did not exist. The need for more effective and practical methods for disposing of spilled hazardous materials and wastes is apparent.

Biological degradation is a common process for disposing of many organic materials, either as a controlled method in biological reaction systems or as a natural method that acts through indigenous populations of microorganisms. Of 650 "designated" hazardous materials, over half are known to be biodegradable (Mercer et al., 1978; U.S. Coast Guard, 1974; Federal Register, 1975, 1978, and 1979). Further research into the metabolic capabilities of microorganisms can be expected to result in the application of biodegradation to a more broad spectrum of hazardous materials. Many chemicals are considered biorefractory simply because the indigenous microorganisms found in conventional biological treatment systems, or in the soil or water affected by a spill, are unable to biodegrade the material. However, other microorganisms may be capable of biodegrading selected biorefractory hazardous materials.

The Atlantic Research Corporation has developed an approach to the problem of ultimate disposal of hazardous materials in which pure cultures of fungi, actinomycetes, bacteria, and yeasts are exposed to so-called biorefractory hazardous chemicals. Each culture is evaluated for its ability to metabolize a specific chemical compound at a realistic rate. The cultures possessing appropriate metabolic capabilities are further tested to determine their potential for the disposal of spilled hazardous materials. The selected cultures must survive in the presence of microorganisms indigenous to the medium in which the spill occurred while still accomplishing the objective of degrading the substance of concern. (It should be noted that indigenous microorganisms may consume organisms that are specially selected and added as inocula.) In the case of spilled hazardous materials where treatment at a central location is not practical, a process for treatment of on-site wastes in needed. Treatment can be an *in situ* process or could be carried out in a mobile unit that would be transported to the site of a spill. In either case, decontamination of the area would be achieved by cultured organisms selected for their ability to metabolize the spilled substances.

This program was a limited feasibility study in which the use of selected pure cultures of microorganisms was evaluated for potential in the biodegradation of some specific, commonly spilled, biorefractory hazardous materials. The limited scope of this project did not allow for selectiion and adaptation of cultures over the long term for biological removal of the selected chemicals. Time constraints and budgetary requirements precluded the use of C-14-labeled chemicals, as well as the extensive chemical analyses required for isolation, identification, and quantification of metabolites and of potential by-products of the biological degradation of the selected chemicals. However, the fraction of the hazardous material that disappeared was corrected for bioaccumulation by the microorganism or for sedimentation or dropout from the medium (the hazardous materials selected had a pH-dependent, limited solubility in the media used).

SECTION 2

CONCLUSIONS

Selected pure culture organisms were evaluated for potential in ameliorating spills or releases of pentachlorophenol (PCP), hexachlorocyclopentadiene (HCCP), and methyl parathion (MP). These three substances are produced and used in quantity, have been spilled on a large scale, are very persistent (except for MP), and cover a spectrum of toxicity and environmental hazards. The following conclusions apply to this study:

1. The pH dependent and limited solubility of these chemicals in water makes evaluation of microbial assimilation and/or biodegradation in an aqueous system difficult.

2. No fully conclusive evidence for the complete biodegradation of PCP, HCCP, or MP by pure culture organisms was obtained in these studies though disappearance was observed and, generally, quantified.

3. The data indicate that a fungal isolate (044) may have potential for degradation and/or assimilation of PCP. A bacterial isolate (041) appeared to reduce the PCP concentration by 22 to 34% when dextrose was provided as a carbon source. This bacterial isolate (041) tolerated higher con- centrations of PCP/oil-in-sandy soil than did the fungal isolate (044). The 041 isolate may have potential for removal of PCP from spill-contaminated areas. The half-time for disappearance ranges from a day to weeks.

4. HCCP disappeared from aqueous solutions; no chlorinated by-products analyses were performed on aqueous media assayed for HCCP removal by microorganisms. There is a further, unresolved ambiguity as to the extent of biodegradation, since the extent of HCCP disappearance was somewhat pH-dependent.

5. Data from MP studies indicate that two microorganisms (003, 021) may have potential for removal of this pesticide when a carbon source such as dextrose is provided.

6. Additives, such as sodium azide, may be required to inhibit the growth of bacteria that preferentially consume food (dextrose, peptone, yeast extract) and thus adversely affect the buildup of the hazardous-material destroying microorganism (isolates).

SECTION 3

RECOMMENDATIONS

Based on the results presented in this report and their potential significance to the cleanup of spilled hazardous materials, the following recommendations are made:

1. Pure cultures of the bacterium 041 plus nutrients and oxygen should be further evaluated for decontaminating pentachlorophenol/oil spill sites.

2. The effectiveness of adapting organisms for complete destruction of pentachlorophenol (PCP), hexachlorocyclopentadiene (HCCP), and methyl parathion (MP) should be evaluated.

3. Future biodegradation studies should emphasize identification of metabolites and the mechanisms of biotransformation or biodegradation. The use of C-14-tagged compounds is recommended as a means of accomplishing this goal.

4. The rate and extent of degradation should be more carefully assessed, by-products and/or products of incomplete biodegradation should be identified, and a larger number of experiments should be undertaken to ensure acquisition of more statistically significant data.

SECTION 4

BIOLOGICAL MATERIALS AND ANALYTICAL PROCEDURES

BIOLOGICAL MEDIA

Stock cultures of the candidate microorganisms were maintained on the following agar media slants:

Difco Sabouraud dextrose agar
BBL trypticase soy agar
ARC No. 50 agar.

Media for the microbial degradation experiments consisted of the basic salts or modified basic salts solution, the hazardous material, a solubilizing agent (when necessary), and a carbon source (dextrose, peptone, or yeast extract). Formulations for the media used in these studies are presented below.

Sabouraud Dextrose Agar (SDA)

Sabouraud dextrose agar (SDA) was used as a selective medium for the cultivation and maintenance of fungi. The dehydrated medium was obtained from BBL Laboratories, Cockeysville, Maryland. Sixty-five grams were added to one liter of distilled water, and the mixture was heated to boiling with constant stirring. Eight ml of this preparation were aliquoted into two hundred 125x16mm screw-cap test tubes. The tubes were autoclaved for 15 minutes at 15 psi. Then the tubes were allowed to cool in a slanted position until the liquid solidified. The medium had the following composition:

Polypeptone™ peptone.....	10.0 g/liter
dextrose.....	40.0 g/liter
agar.....	15.0 g/liter

Trypticase Soy Agar (TSA)

Trypticase soy agar (TSA) was used as a general purpose medium. The dehydrated product was supplied by BBL Laboratories. Forty grams of dehydrated medium were dissolved in one liter of distilled water. Slants were prepared as described for Sabouraud dextrose agar. The final composition of the prepared medium was:

trypticase peptone.....	15.0 g/liter
phytone peptone.....	5.0 g/liter
sodium chloride.....	5.0 g/liter
agar.....	15.0 g/liter

ARC No. 50 Medium

ARC No. 50 medium was used as a non-specific agar and liquid medium. The agar slants were prepared as for Sabouraud dextrose agar. Liquid No. 50 was prepared by omitting the agar constituent. The composition of this medium was:

K_2HPO_4.....	1.0 g
KH_2PO_4.....	1.0 g
NH_4NO_3.....	1.0 g
yeast extract (Difco).....	2.0 g
solutions of:	
$CaCl_2$ (0.027 g/ml).....	1.0 ml
$MgSO_4$ (0.022 g/ml).....	5.0 ml
$FeCl_3$ (0.00035 g/ml).....	2.5 ml
agar (Difco).....	15.0 g
distilled water (to make)..	1.0 liter

Basic Salts (B.S.) and Modified Basic Salts (M.B.S.)

Basic salts (B.S.) and modified basic salts (M.B.S.) solutions were used as the experimental test media. The composition of the salts media is given below. The final pH of the basic salts medium was 6.8 and that of the modified basic salts was 8.3 - 8.5. Depending on the experimental design, either dextrose, peptone, or yeast extract was added to the basic salts or modified basic salts medium.

Chemical	Basic Salts medium	Modified basic salts medium
KH_2PO_4.....	1.0 g	none
K_2HPO_4.....	1.0 g	2.0 g
NH_4NO_3.....	1.0 g	1.0 g
NaOH.....	as required	as required
Solutions of:		
$CaCl_2$ (0.027 g/ml).....	1.0 ml	1.0 ml
$MgSO_4$ (0.022 g/ml).....	5.0 ml	5.0 ml
$FeCl_3$ (0.0025 g/ml).....	1.0 ml	1.0 ml
Distilled water (to make)...	1.0 liter	1.0 liter

TEST MICROORGANISMS

The pure culture microorganisms used in these studies are listed and described in Table 1, which also shows the hazardous material added to challenge the microorganism. The number of organisms tested was limited by the funding of the contract; and the selection was based on known performance and source data for the microorganisms. The original selection was reviewed by a panel of biochemists and biologists who recommended some substitutions.

TABLE 1. TEST MICROORGANISMS

Organism	Source	Medium	Description
Hexachlorocyclopendatiene			
006	Bagasee	TSA	Bacterium. Small, white mucoid colony, Gram-negative rod.
016	Soil	SDA	Fungus. White with green center. Aerial mycelia.
020	Soil	SDA	Fungus. Black with white aerial mycelia that are very long, septate and thick. Terminal macroconidia in chains. Microconidia in groups along mycelia.
022	Soil	SDA	Believed to be a fungus. No aerial mycelia. White, stringy colonies. Huge oblong, rectangular Gram-positive rods with connecting branches (non-staining).
QM 9123 (ARC 123)	QM-Natick Labs, Mass.	SDA	Subculture of a Trichoderma viride. Grayish-white fuzzy colonies. Branched, non-septate mycelia with some micro-conidia.
ATCC 1369 (ARC 369)	American Type Culture Collection	TSA	Yeast. Subculture of Candida tropicalis. Round convex, off-white, dullish colonies. Gram-positive ovals with budding in clusters.
ATCC 21505 (ARC 505)	American Type Culture Collection	TSA	Actinomycete. Nocardia globerula. Tiny round off-white convex colonies. Gram-positive cocci-rods diploid.
Methyl Parathion			
003	Septic tank seed	TSA	Bacterium. Gram-positive cocci in clusters.
010	Cotton seed bulls	TSA	Fungus. Fuzzy white with black tips. Long non-septate mycelia.
016	Soil	SDA	Fungus. White with green center and aerial mycelia.

(Continued)

TABLE 1 (continued)

Organism	Source	Medium	Description
Methyl Parathion (continued)			
020	Soil	SDA	Fungus. Black with white aerial mycelia that are very long, septate, and thick. Terminal macroconidia in chains. Microconidia in groups along mycelia.
021	Soil	SDA	Fungus. Yellow to tan with aerial mycelia. Long non-septate mycelia with macro- and microconidia. Macroconidia are not terminal.
022	Soil	SDA	Believed to be a fungus. White stringy colonies. Huge oblong, rectangular Gram positive rods with connecting branches (non-staining).
026	Newspaper	#50	Fungus. White on edges,green in center, little or no aerial mycelia.
QM 9123	QM-Natick	SDA	Subculture of a Trichoderma viride. Grayish white fuzzy colonies. Branched, non-septate mycelia with some microconidia.
ATCC 9645 (ARC 645)	American Type Culture Collection	#50	Subculture of a Gliocladium virens.
ATCC 9950 (ARC 950)	American Type Culture Collection	SDA	Subculture of Candida utilis. Colonies round, convex, mucoid, off-white with a banana-like smell. Gram positive ovals with budding.
Pentachlorophenol			
004	Septic Tank seed	#50	Fungus. White aerial mycelia that are long, branched, septate. No conidia.
016	Soil	SDA	Fungus. White with green center with aerial mycelia.
026	Newspaper	#50	Fungus. White on edges, green center, little or no aerial mycelia.

(Continued)

TABLE 1 (continued)

Organism	Source	Medium	Description
Pentachlorophenol (continued)			
040	PCP medium contaminant	TSA	Bacterium. Off-white, circular, convex, smooth colonies. Gram-positive cocci.
041	PCP medium contaminant	#50	Bacterium. Off-white, irregular colonies forming a convex, rough, convoluted colony. Gram-positive cocci.
042	PCP medium contaminant	TSA	Bacterium. Off-white, very small colonies forming a regular, convex surface. Gram-positive rods.
043	HCCP medium contaminant	SDA	Bacterium. Very mucoid, off-white, fast growing. Gram-positive cocci in clusters.
044	HCCP medium contaminant	SDA	Fungus. White and fuzzy with aerial mycela. Long branched, septate mycelia. No conidia.
045	PCP medium contaminant	TSA	Off-white medium-sized colonies. Slightly irregular and rough, but not grainy. Gram-negative rods.

ANALYTICAL PROCEDURES

Pentachlorophenol (PCP)

Products from initial laboratory screening tests (Refer to Section 5) were analyzed for residual PCP using a High Performance Liquid Chromatograph (HPLC). The procedure used was as follows:

1. A 100 ml sample was extracted with 50 ml hexane.
2. The aqueous fraction was re-extracted with 50 ml hexane.
3. Water was removed from the hexane extract by adding anhydrous Na_2SO_4.
4. An aliquot (1 or 2 ml) of the hexane extract was evaporated to dryness.
5. The residue was dissolved in 50 ml of water and diluted to 100 ml with acetonitrile.
6. The sample thus prepared was filtered through a 0.45-u Millipore Fluoropore filter. An aliquot of the filtrate was then injected into the HPLC.

Analysis was performed on a Waters Model 440 HPLC using a micro Bondapak C-18 column. An acetonitrile:water mixture (60:40,V:V) was used as the carrier. The carrier fluid was pumped through the HPLC unit on a once-through basis. The sample was injected into the carrier fluid as a means of transporting it to the separation column and the detector.

In later tests, electron capture gas chromatography (GC-EC) was used to quantitate PCP after derivatization. The procedure, shown schematically in Figure 1, was similar to that of Rudling (1970). Prior to GC-EC analysis, the acetyl derivative of PCP was prepared using the following procedure. One hundred ml of aqueous PCP-containing medium was adjusted to pH 11 by addition of 1N NaOH.

The resulting Na-PCP solution was transferred to a 200 ml volumetric flask to which were added 1 ml of acetic anhydride and 10 ml of hexane. The contents were mixed vigorously for 30 minutes. Distilled water was added and the layers allowed to separate. The hexane layer, containing the acetate derivative of PCP, was removed. Frequently, the biomass formed an emulsion in the hexane layer. To break this emulsion, the hexane layer was stored at -20°C.

The hexane layer was diluted with pesticide-grade hexane in order to achieve concentrations that could be assayed by GC-EC. A Hewlett-Packard Model 554A gas chromatograph with a ^{63}Ni detector was used for the analyses. The column was 10% DC-200 on 80/100 Chromosorb WHP, and the oven temperature was 230°C. A 5%-methane/95%-argon mixture was used as the carrier gas at a flow rate of 30 ml/min.

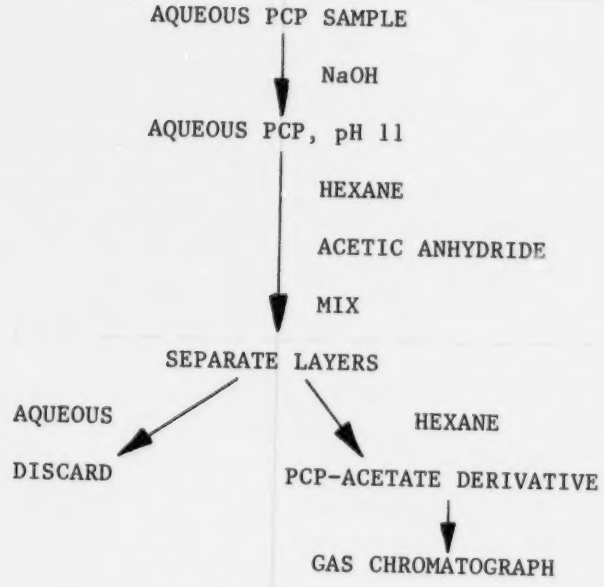

Figure 1. Scheme for PCP derivatization and analysis.

Purification of the PCP-derivative was accomplished by successive recrystallization from hexane. The purity of the final product was 98+ %. A standard stock solution of 917.0 ppm PCP-acetate in hexane was prepared. From this stock the following standards were prepared: 1.0, 0.75, 0.50, 0.25, 0.125, and 0.0 ppm.

A similar protocol was followed to quantitate the amount of PCP stored in or bioaccumulated by the biomass. Where practical, correction was made separately for the PCP that dropped out of solution.

Hexachlorocyclopentadiene (HCCP)

A 100 ml aliquot of aqueous HCCP medium was extracted twice with 40 ml portions of hexane in a separatory funnel. The aqueous portion was discarded, and the hexane layers pooled in a 100 ml volumetric flask and brought to volume with additional hexane.

HCCP samples were analyzed by two methods: (1) High Performance Liquid Chromatography (HPLC) and (2) Electron Capture Gas Chromatography (GC-EC). For the HPLC analysis, the extract was dilulted in acetonitrile (CH_3CN) and filtered through 0.45-u Millipore Fluoropore filters in a 13-mm Swinney stainless steel filter holder. The assay for HCCP content was performed on the Waters Model 440 HPLC with a micro Bondapak C_{18} column. The detector wave length was set at 254 nm. One hundred percent reagent-grade acetonitrile was used as the carrier at a flow rate of 2.0 ml/min. With injection volumes of 5 ul, HCCP was detectable in the 10 to 100 ppm range. A standard stock solution of HCCP was prepared by diluting 0.3 ml of HCCP with 100 ml pesticide-grade hexane to achieve a concentration of 5000 mg/l. Working standards containing 100, 50, 25, and 10 ppm HCCP were prepared daily by diluting the 5000 ppm stock solution with glass-distilled acetonitrile.

Hexachlorocyclopentadiene samples were prepared for gas chromatographic analysis by extraction of the aqueous media as described above. These extracts were diluted with pesticide-grade hexane. Analyses were performed on a Hewlett Packard Model 554A gas chromatograph with a 10% DC-200 on 80/100 Chromosorb WHP column. Oven temperature was maintained at 205°C. A 5%-methane/95%-argon mixture was used as the carrier gas at a flow rate of 40 ml/min. Using this method, concentrations of HCCP down to the 0.2 to 1 ppm range were quantifiable. Some problems were encountered in the solubilizing of HCCP in the medium and in measuring the amount of HCCP bioaccumulated or precipitated.

Methyl Parathion (MP)

Methyl Parathion 500 was obtained from Monsanto Company. Its composition was as follows:

Active Ingredients:	
0,0-Dimethyl O-p-nitrophenyl phosphorothioate*	46.8%
Related compounds	2.4
Aromatic petroleum derivative	44.4
Inert Ingredients	6.4
	100.0%

The procedures for quantitative analyses were based on the work of Jackson (1975). Since the solubility of MP in water is limited, a standard stock solution was prepared by diluting Methyl Parathion 500 with absolute methanol to 1000 ppm MP. Working standards were prepared on a daily basis by diluting the stock solution to 500 ppm with distilled water. Other working standards (100, 50, 25, 10, and 5 ppm MP) were prepared by diluting the stock solution with a 50:50 mixture of absolute methanol:water.

Analyses were performed on a Waters Model 440 HPLC with a micro Bondapak C_{18} column. The carrier phase was an 80:20 mixture of methanol:water. With 10-µl injections, MP was quantifiable in the 10 to 200 ppm range.

In order to substantiate the composition of the Methyl Parathion 500 solution, a purified sample of MP was obtained from Mr. Lloyd Boesch of Monsanto. Solutions of known concentrations were prepared by dissolving the purified material in 50:50 absolute methanol:water mixtures. Quantitative comparisons between pure MP and the Methyl Parathion 500 standard solutions were made. The Methyl Parathion 500 solutions proved to be quantitatively correct and were used to prepare standard curves for MP analysis.

Experimental samples containing MP were prepared for HPLC analysis by the procedure given below:

1. An aliquot of the aqueous medium was mixed with an equal volume of glass distilled methanol and vigorously shaken.

2. The mixture was filtered through a 0.45-u Millipore Fluoropore filter.

3. Filtrates were placed in clean, screw-cap vials for analyses by HPLC.

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* Equivalent to 4.2 lb of 100% methyl parathion per gallon (500 g/l).

SECTION 5

EXPERIMENTAL METHODS

LABORATORY SCREENING TESTS

A series of screening tests was conducted to identify pure culture organisms capable of degrading selected hazardous substances. The hazardous material was dissolved or suspended in a sterile aqueous medium in 250-ml Erlenmeyer flasks. Each organism was grown in 50 ml of the liquid medium specified in Section 4. The entire culture was centrifuged and the cell pellet resuspended in 10 ml sterile saline solution. Each flask was inoculated with the resuspended cell pellet of the selected organism as indicated for each experiment. A list of organisms used in the laboratory screening tests is presented in Section 4. The flasks were plugged with cotton and placed on a rotary shaker, which provided the necessary aeration during the test period. All flasks were maintained at room temperature.

The flasks were visually inspected for turbidity at least on a daily basis to assess microbial growth. Chemical analyses of the supernatant and the cell mass were performed on those flasks that contained viable organisms. Organisms that appeared to degrade the hazardous chemical were used for further tests in which nutrient formulations or test parameters, such as pH, were examined.

The details of the actual laboratory screening tests and a discussion of the data obtained are presented in Section 7.

PILOT-SCALE TESTS

Degradation of pentachlorophenol (PCP) was examined on a pilot scale in both batch and continuous tests. The apparatus and test methodology are described below.

The details of the actual pilot-scale work and a discussion of the results obtained are presented in Section 8.

Batch Tests

Batch tests were performed in 40-liter (10-gallon) glass tanks with approximately 16 liters (four gallons) of feedstock treated in each tank. Constant aeration was provided by a series of aeration tubes in the bottom of the tanks. Dissolved oxygen, pH, and temperature were monitored during each test. Samples were withdrawn at regular intervals for chemical analysis, which was performed by GC-EC as described in Section 4, Analytical

Procedures. The details on number, purpose, and duration of the batch tests are presented in Section 8. Batch testing was chiefly employed in evaluating the use of sodium azide to inhibit bacterial growth in fungus-inoculated PCP-pilot-scale degradation tests.

Continuous Tests

Pilot-Scale Facility Description--A continuous reactor unit, illustrated schematically in Figure 2, was constructed for pilot-scale tests. The reactor was designed to process 1 kg (2.2 pounds) of influent per hour with an average residence time of 100 hours. The unit holds a liquid volume of approximately 100 liters (25 gallons). To promote continuous, but sequential, contact with organisms, the tank was divided into four long, narrow compartments that were sized to ensure efficient aeration and mixing. Each compartment had a capacity of approximately 24 liters (6 gallons). Photographs of the reaction unit are shown in Figures 3 and 4.

The tank and lid were constructed of acrylic sheet (Plexiglas) panels bonded with PS-30 acrylic cement. The dimensions of the unit were 81 cm (32 in.) long x 40 cm (16 in) wide x 63 cm (25 in.) high. Clear Plexiglas was selected for its strength and because it permitted visual monitoring of the unit during operation. Acrylic tubing was used to form influent, effluent, and air supply ports through the chamber walls. Tygon tubing was used for the flexible air and liquid transport lines. The unit was made pressure tight with a neoprene-covered seal gasket that contacted the lid when closed. Eight latches were used to maintain pressure on the seal. A similar seal was used on the sludge collector (separator) cover, but the lid was held down by elastic bands. The separator was also constructed of acrylic and measured 36 cm (14 in.) in height x 15 cm (6 in.) in diameter.

A peristaltic pump was used to meter the feed and the sludge return to the unit. The motor speed was maintained at 50 rpm, delivering approximately 1 kg (2.2 pounds) of influent per hour through the larger of two pumps and returning approximately 0.4 kg (0.8 pounds) per hour of sludge through the smaller pump. By-pass tubes were used to adjust the actual flow delivered to the processing chamber.

Each compartment was equipped with two parallel aeration tubes (air wands) located at the bottom of the unit. These tubes allowed infusion of filtered air (supplied by a compressor) and provided mixing. Individual compartment sampling ports were located on alternating ends of the unit. Feedstock from the 40-liter (10-gallon) reservoir entering the first compartment was well mixed and continuously aerated. Liquid from the first stage overflowed through a V-notch into the second stage, was mixed and aerated, then flowed similarly into the third stage, and onto the fourth stage. The fourth stage overflowed into the sludge collector-separator. A baffle located near the point of entry into the sludge collector-separator helped promote settling of the solids.

The main processing chamber and sludge collector were mutually pressurized by the aeration supply. The elevation of the effluent tube from the main chamber overflow into the collector and the resistance in the air

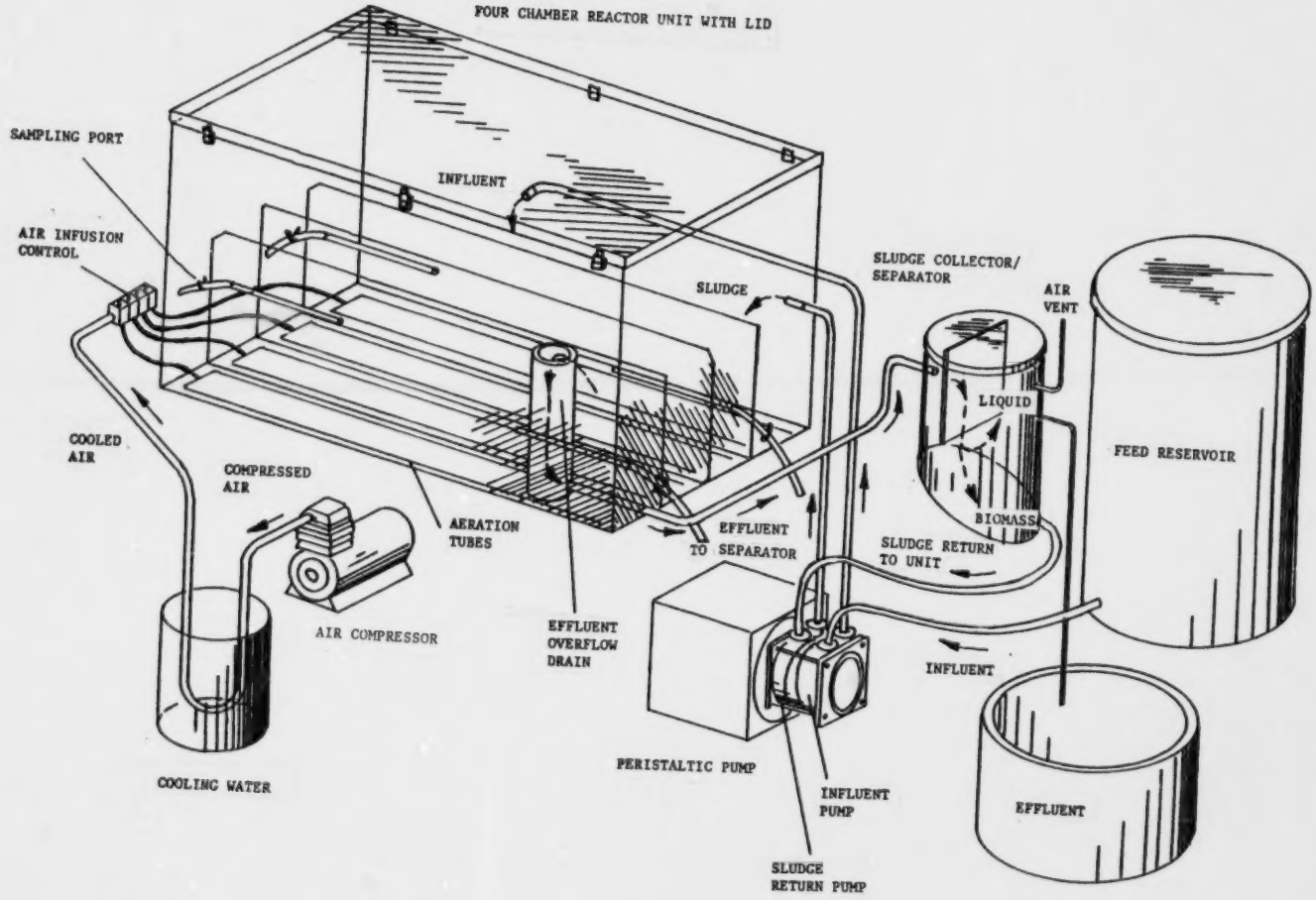


Figure 2. Schematic diagram of continuous pilot-scale reactor.

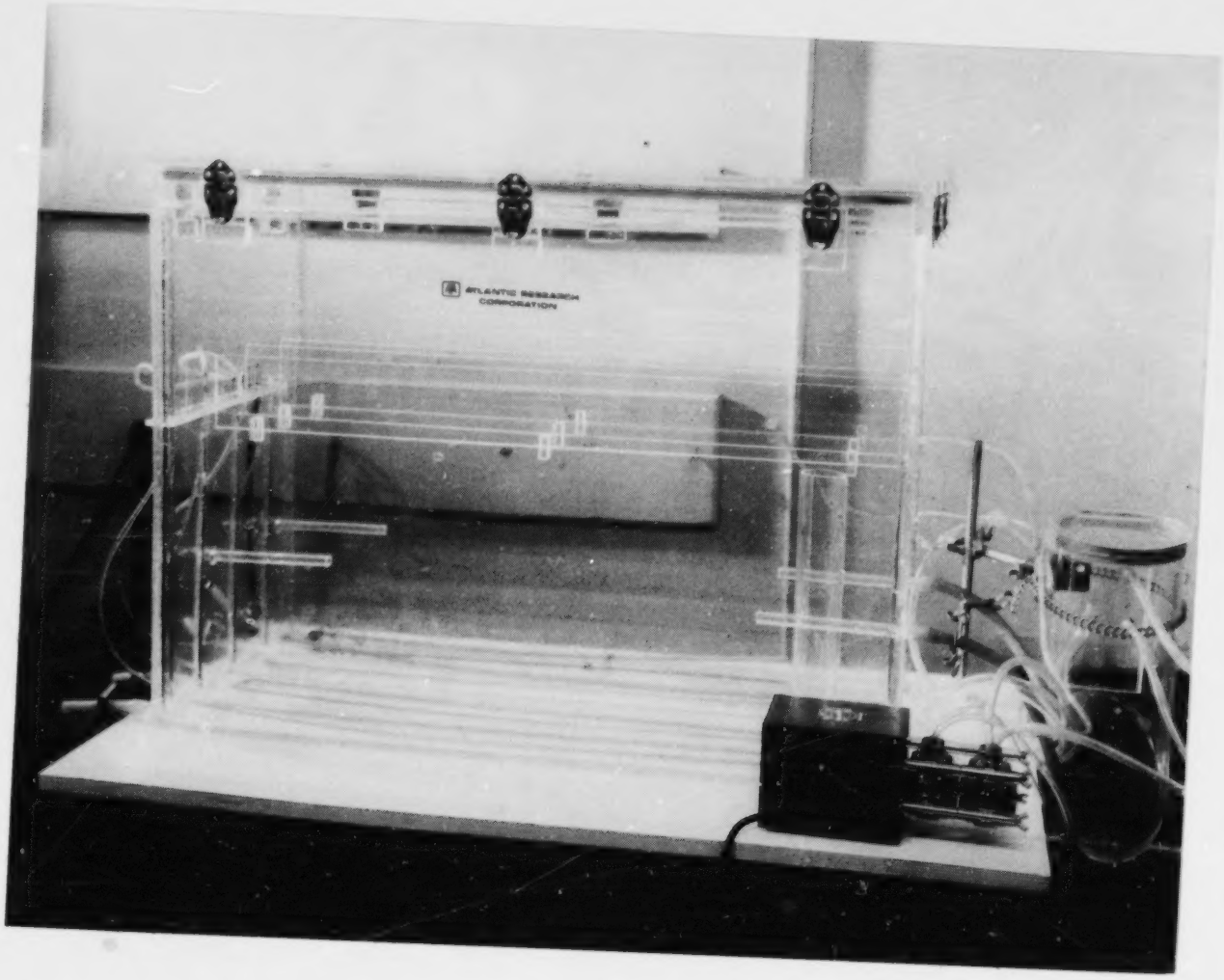


Figure 3. Continuous pilot-plant unit fabricated from poly(methylmethacrylate) sheets with overall dimensions (inches) of 32 (wide) x 25 (high) x 16 (deep), showing clamped, air-tight, removable cover (top), three separator plates (front-to-rear) that divide the compartment into 4 chambers and also serve as weirs, air infusion "wands" (bottom of chambers), sampling ports (left and right sidewalls), overflow drain (right front corner), two-chamber peristaltic pump (right foreground) that provides inflow of fluid and sludge return, and sludge collector/reservoir (right rear).

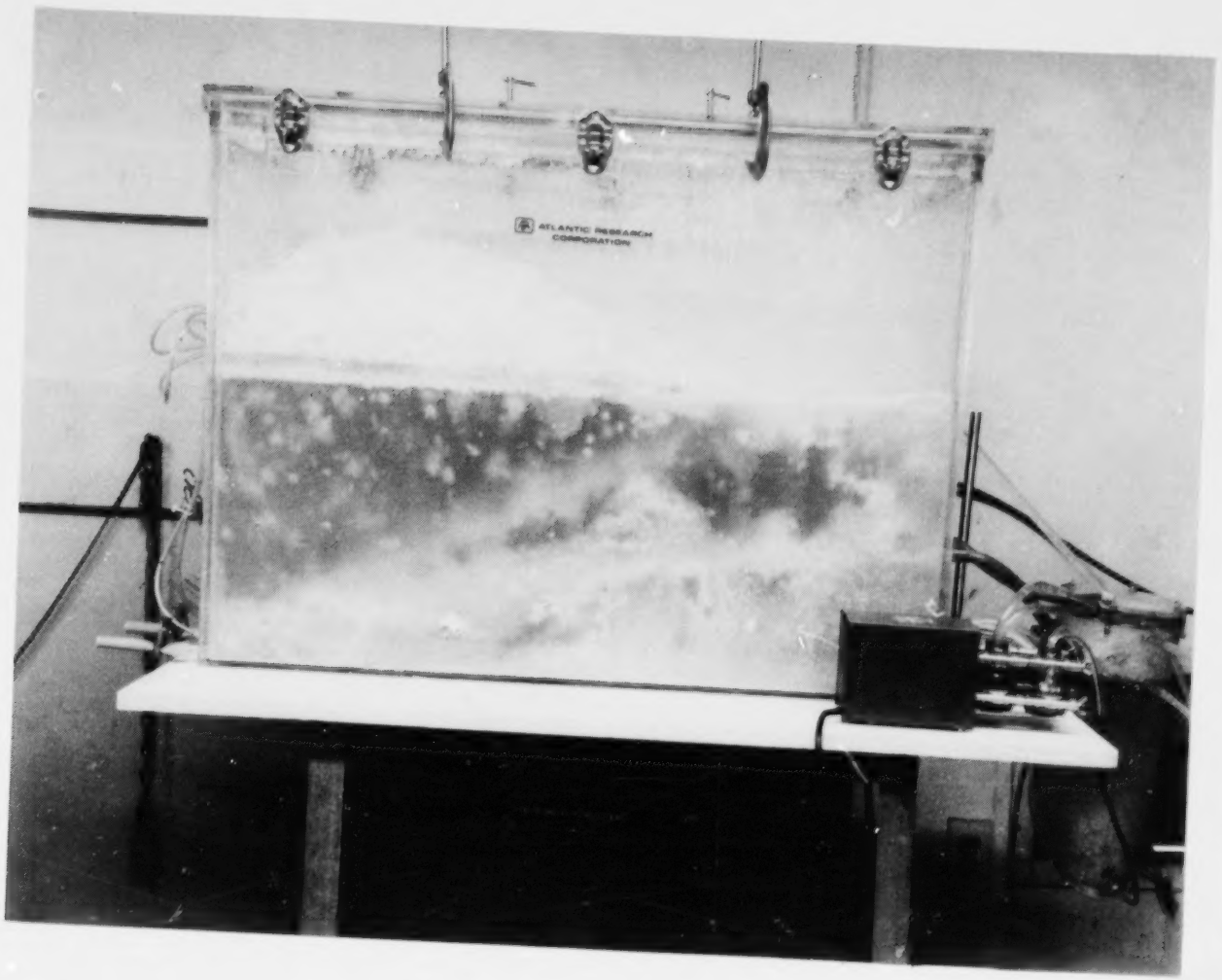


Figure 4. Continuous pilot plant unit on 8th day of operation (no pollutants present) showing buildup of fungus within and above chambers. (Note "puff ball" appearance of fungus.)

exhaust tube filter resulted in an internal pressure of approximately 13 cm (5 in.) of water.

Two preliminary runs were made in order to standardize aeration and feed rates. The chambers were filled with a medium consisting of basic salts solution and 0.2% dextrose and were seeded with organism 016 as follows:

Chamber 1 - 800 ml inoculum
Chamber 2 - 500 ml inoculum
Chamber 3 - 300 ml inoculum
Chamber 4 - 300 ml inoculum.

Several problems were encountered during the first test. These problems were corrected and a second test was initiated. In the second test, the unit was operated for 8 days with a feed rate of approximately 54 liters (13.5 gallons) per day. Throughout the run all components operated smoothly with only minor adjustments required.

Pilot-Scale Test Procedures--The four test chambers were filled with the following medium at a final pH of 8.5, which the screening tests had shown to be appropriate.

Compound	Quantity
K_2HPO_4	60.8 g
NH_4NO_3	30.4 g
$CaCl_2$	0.6 g
$MgSO_4$	6.1 g
$FeCl_3$	0.02 g
Dextrose (0.2%).....	121 ml
PCP	6.05 g
1N NaOH	150 ml
Deionized Water	62 liter

Then the chambers were inoculated with the microorganism previously grown in ARC No. 50 medium. The initial distribution of microbial cells was:

Chamber 1..... 1300 ml of microbial culture
Chamber 2..... 500 ml of microbial culture
Chamber 3..... 500 ml of microbial culture
Chamber 4..... 0 ml of microbial culture.

Total continuous running time was 14 days for most tests. The feed rate was maintained at approximately 2.3 liters (0.6 gallons) per hour. Visual inspection was maintained on a daily basis, and analytical tests were conducted on samples from the four chambers. Temperature, pH, and DO were measured, the latter with a YSI Model 54A Oxygen Meter.

MICROBIAL TESTING OF SAMPLES FROM AN ACTUAL, SPILL-CONTAMINATED SITE

A description of the site, the details of the tests performed, and a discussion of the results obtained are presented in Section 9.

SECTION 6

PROPERTIES OF THE HAZARDOUS MATERIALS SELECTED FOR STUDY

The goal of this program was to demonstrate the feasibility of utilizing pure culture microorganisms for ultimate disposal of spilled hazardous materials. The technical approach included:

- o selection of the hazardous materials to be evaluated.
- o performance of a literature survey on each selected chemical to identify previous biodegradation studies, analytical methodologies, and specific handling and disposal problems
- o formulation of an aqueous medium, suitable for microbial growth, containing the hazardous material in solution.
- o development of analytical procedures, including extraction and assay techniques.
- o selection of microorganisms for biodegradation studies through a series of laboratory screening tests.
- o construction of a continuous pilot-scale reactor unit for large-scale studies.
- o performance of batch and continuous pilot-scale studies.
- o analysis of test results.

These aspects are covered partly in this Section and partly in the following Sections (7,8,9).

SELECTION OF HAZARDOUS MATERIALS

A list of potential chemicals for experimental work was drawn from the 650 "designated" hazardous chemicals cited in the Federal Register (1975, 1978, and 1979) and the CHRIS list (U.S. Coast Guard, 1974). The selection of the chemicals to be studied during this program was based on:

- o the severity of impact of spills
- o biodegradability

- toxicity
- general chemical class.

The following chemicals were selected for study:

- pentachlorophenol (PCP)
- hexachlorocyclopentadiene (HCCP)
- methyl parathion (MP).

The selection of PCP was based on the following factors:

- high annual U.S. production, e.g., 52.4 million lb. in 1974 (U.S. International Trade Commission, 1975)
- widespread use of PCP as a pesticide
- toxicity to mammals and aquatic organisms
- potential environmental hazards
- a current PCP spill problem in Haverford, Pennsylvania
- high environmental persistence.

The bases for selecting HCCP follows:

- high volume chemical intermediate, e.g., 50 million lb. in 1975 (U.S. International Trade Commission, 1976)
- little knowledge of environmental fate
- high environmental persistence
- involvement in a recent spill in Louisville, Kentucky.

The selection of MP was based on the following factors:

- toxic, non-chlorinated pesticide
- produced in large quantities
- initially disastrous effects on the environment
- partially detoxified by in-situ hydrolysis.

PCP and HCCP are representative of environmentally persistent chlorinated compounds, pesticides, and their intermediates. Other types of pesticides, such as the organophosphates and carbamates, are not so persistent as the chlorinated compounds. However, in a spill situation, the highly toxic organophosphates and carbamates can have a disastrous effect on the environment. Therefore, it was decided to include one of these pesticides in the study. MP, an organophosphate insecticide, was selected.

PROPERTIES OF THE SELECTED HAZARDOUS SUBSTANCES

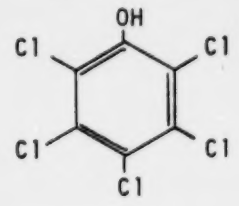
Prior to initiating laboratory studies of the selected compounds, a literature search was performed. The goals of this literature search were to identify:

- o previous environmental studies of these compounds
- o reliable analytical methods
- o specific handling and disposal procedures and problems

A summary of the literature search by chemical follows.

Pentachlorophenol (PCP)

Physico-chemical Properties--Pentachlorophenol has the molecular formula C_6HOCl_5 corresponding to the following structure:



Selected properties of this compound are:

Molecular weight:	266.34
Melting point:	190-191 ^{o}C (anhydrous)
Boiling point:	309-310 ^{o}C (with decomposition)
Specific gravity	1.978 (at 22 ^{o}C compared to H_2O at 4 ^{o}C)
Solubility:	under neutral conditions, slightly soluble (14 mg/l) in cold water; solubility decreases with decreasing pH; under alkaline conditions solubility increases because of formation of the more soluble sodium salt, sodium pentachlorophenate (Na-PCP).

Use and Manufacture--Pentachlorophenol is a widely used pesticide employed as a fungicide, herbicide, defoliant, insecticide, and wood preservative. It is marketed under a variety of trade names including: DOWICIDE™7 (DOW Chemical), Penta General Weed Killer™ (Chipman), and Santobrite™ (Monsanto).

Pentachlorophenol is manufactured by one of two processes:

- o direct chlorination of molten phenol in the presence of a catalyst
- o alkaline hydrolysis of the appropriate chlorobenzene.

Direct chlorination of phenol is the most widely used preparatory method for PCP because it results in fewer unwanted by-products.

Pentachlorophenol formulations sold in the United States contain several impurities. These impurities include tetrachlorophenol, chlorinated dioxins (1-2000 ppm), chlorinated diphenyl ethers (100-1000 ppm), chlorinated dibenzofurans (50-200 ppm), and chlorinated phenoxyphenols (1%) (Nilsson et al., 1977).

Of particular environmental concern are the chlorinated dioxins and the chlorinated phenoxyphenols ("predioxins"). Chlorinated dioxins, which are very toxic, can be formed from chlorinated phenols when high temperatures occur during the manufacturing process. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most toxic chemicals known to man. The acute oral LD_{50} of TCDD for mice is less than 100 ug/kg (NIOSH, 1977). Both the mono- through tri-chlorodibenzo-p-dioxins and the other isomers of the tetra-chloro compound are less toxic than TCDD. More fully chlorinated dioxins are also less toxic than TCDD; however, the potential exists for environmental dechlorination to TCDD.

The toxicological and environmental problems associated with dioxin impurities were recognized by PCP manufacturers in the late 1960's. Considerable effort has been expended by U.S. manufacturers to clean up the final PCP product. Analyses of a commercially available biocide PCP (DOWICIDE EC-7™) were performed by Schwetz et al., (1977). The results of these analyses are presented in Table 2. DOWICIDE EC-7 is representative of the purified grades of PCP. Technical grades currently made in the U.S. and abroad have higher concentrations of non-phenolic impurities. PCP products made before 1972 have significant quantites of non-phenolic impurities.

Toxicological Properties--The toxicological properties of pentachlorophenol and its sodium salt are well documented (Sax, 1979; Fairchild, 1977). As shown in Table 3, these compounds are toxic by all routes of administration. However, the greatest acute toxicity occurs via inhalation. The physiological injury for acute doses of pentachlorophenol is centered mainly on the vascular system, with death resulting from heart failure (NIOSH, 1977). Heart failure is a result of the ability of pentachlorophenol to uncouple oxidative phosphorylation and alter the electrical conductivity of cellular membranes (Dougherty, 1977). A threshold limit value (TLV) for pentachlorophenol of 0.5 mg/m^3 in air has been recommended in order to protect workers (ACGIH, 1977).

In studies with mice, Innes et al., (1969) reported no significant tumorogenicity when these mammals were fed the maximum tolerable PCP dose of 46.4 mg/kg/day. However, there are conflicting reports concerning the mutagenicity of pentachlorophenol (Dougherty 1977). The conflict may in part be due to the presence of mutagenic impurities, such as the chlorinated dioxins, in the pentachlorophenol.

Pentachlorophenol and its sodium salt are highly toxic to aquatic organisms, as shown by the results of bioassay tests of PCP and Na-PCP,. presented in Table 4. In general, fish and oysters are very sensitive to

TABLE 2. ANALYSIS OF PENTACHLOROPHENOL (DOWICIDE EC-7)*

Component	Concentration
Phenolic components:	
Pentachlorophenol	90.4±1.0%
Tetrachlorophenol	10.4±0.2%
Trichlorophenol	0.1%
Nonphenolic components:	
Dibenzo-p-dioxins:	
2,3,7,8-tetrachloro-	0.05 ppm**
hexachloro-	1.0±0.1 ppm
heptachloro-	6.5±1.0 ppm
octachloro-	15.0±3.0 ppm
Dibenzofurans:	
hexachloro-	3.4±0.4 ppm
heptachloro-	1.8±0.3 ppm
octachloro-	1 ppm**
Hexachlorobenzene	400±40 ppm

* Source: Schwetz et al., 1977.

** Concentration less than level of sensitivity of the analytical method used in 1977.

TABLE 3. ACUTE MAMMALIAN TOXICITY OF PENTACHLOROPHENOL AND SODIUM PENTACHLOROPHENATE (NIOSH, 1977)

Compound	Administration	Species	Dosage (mg/kg)	Response
PCP	Oral	Human	29	LD_{50}
PCP	Oral	Rat	50	LD_{50}
PCP	Skin	Rat	105	LD_{50}
PCP	Skin	Rat	56	LD_{50}
PCP	Subcutaneous	Rat	100	LD_{50}
PCP	Inhalation	Rat	11.7	LD_{50}
Na-PCP	Oral	Rat	210	LD_{50}
Na-PCP	Subcutaneous	Rat	72	LD_{50}

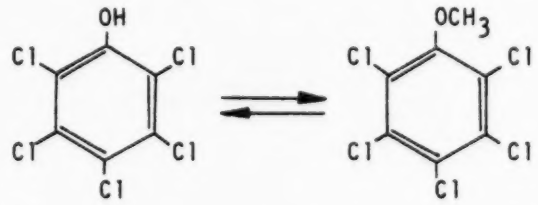
TABLE 4. ACUTE TOXICITY OF PENTACHLOROPHENOL AND SODIUM PENTACHLOROPHENATE TO AQUATIC ORGANISMS

Species	Size	Type of test	Chemical	Temp °C	Salinity ‰	96-hr LC_{50} ppb	Reference
Sheepshead minnow (Cyprinodon variegatus)	1-day fry	Static	PCP	30	10	329	Borthwick and Schimmel, 1977
	2-week fry	Static	PCP	30	10	392	"
	4-week fry	Static	PCP	30	10	240	"
	6-week fry	Static	PCP	30	10	223	"
Pinfish (Lagodon rhomboides)	48-hour prolarvae	Static	Na-PCP	20	26	38	"
Longnose killifish (Fundulus similis)	80mm	Flow-through	Na-PCP	25.0	20.8	53.2	Schimmel et al., 1977
	42mm	Flow-through	Na-PCP	24.4	22.9	306	"
Bluegill (Lepomis macrochirus)	-	Static	Na-PCP	-	-	20	Inglis and Davis, 1977
Goldfish (Carassius auratus)	-	Static	Na-PCP	-	-	50	"
Grasss shrimp Palaemonetes pugio	24-hour larvae	Static	Na-PCP	25	24	649	Borthwick and Schimmel, 1977
	18 mm	Flow-through	Na-PCP	24.8	24.3	515	"
Oyster (Crassostrea virginica)	45mm	Flow-through	Na-PCP	8.4	20.3	76.5	Schimmel et al., 1977

Na-PCP and PCP. However, many invertebrates other than oysters are resistant to these chemicals (Goodnight, 1942).

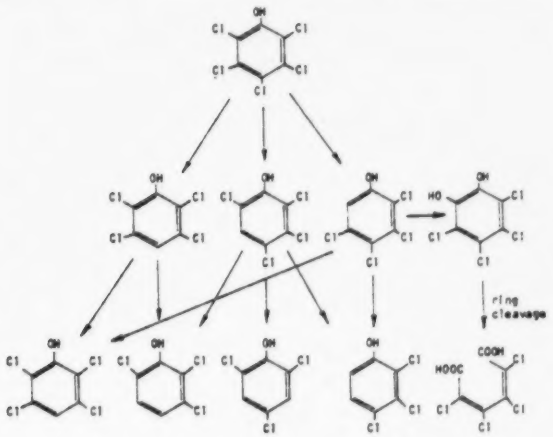
Environmental Fate--The environmental photochemistry of PCP at various concentrations and pH values has been studied by several investigators. Dilute aqueous solutions (100 ppm) of Na-PCP were degraded by sunlight or UV light to less substituted chlorophenols, tetrachlorodihydroxybenzenes, and dichloromaleic acid, the latter resulting from ring cleavage. Continued irradiation produced hydroxylated trichlorobenzoquinones, trichlorodiols, and increased amounts of dichloromaleic acid (Wong and Crosby, 1977). Wong and Crosby also observed the formation of octachlorodibenzo-p-dioxin upon irradiation of more concentrated Na-PCP solutions. However, 2, 3, 7, 8-tetrachlorodibenzeno-p-dioxin was not detected in similar experiments (Crosby et al., 1971). Other investigators reported that Na-PCP solutions were degraded by sunlight to tetrachlororesorcinol and chloranilic acid, with concurrent formation of chlorinated phenoxybenzoquinone dimers and a trimer (Kuwahara et al., 1966). Thus, photochemical decomposition appears to be a significant pathway for transportation/decomposition of PCP in the environment.

Degradation of PCP by soil and selected culture microorganisms has been reviewed by Kaufman (1977). Available data indicate that there are two major pathways for microbial degradation of PCP. These pathways, shown in Figure 5, involve either reductive or oxidative dehalogenation at the ortho and/or para ring positions, and can lead to ring cleavage with CO_2 evolution. The reductive pathway predominates in soils, while the oxidative pathway is common in isolated cultures of microorganisms, such as Trichoderma sp. and Pseudomonas sp. In addition to the two degradation pathways, PCP can also be transformed via methylation to pentachloranisole, which is highly resistant to degradation. This reaction is illustrated below:



Analytical Methodology--The most widely used technique for detecting PCP in the environment and biological samples involves cleanup (processing) of the sample, derivatization of the PCP, and analysis of the derivative using electron capture gas chromatography (GC-EC). Two PCP derivatives are widely employed for this purpose: (1) pentachloroanisole, formed by the reaction of diazomethane with PCP (Bevenue et al., 1966), and (2) pentachlorophenyl-acetate, formed by acetylation of PCP with acetic anhydride (Rudling, 1970). Use of either of these methods enables detection of PCP in the ppb range.

Reductive Dehalogenation (adapted from Kaufman, 1977)



Oxidative Dehalogenation (adapted from Reiner *et al.*, 1977)

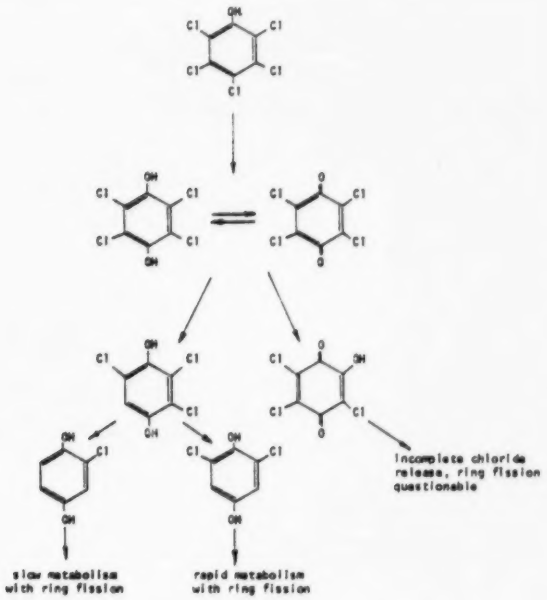
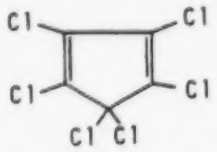

incomplete chloride release, ring fission questionable
slow metabolism with ring fission
rapid metabolism with ring fission

Figure 5. Pathways for microbial degradation of PCP.

The sensitivity and selectivity of negative chemical ionization mass spectrometry (Dougherty and Hett, 1977) and ultraviolet ratio spectrophotometry (Fountaine et al., 1975) have also proven to be satisfactory for detecting PCP. Chemical ionization mass spectrophotometry is sensitive to PCP levels as low as 1 ng. Ultraviolet ratio spectrophotometry is capable of detecting PCP in water in the 10 to 500 ppb range.

Hexachlorocyclopentadiene

Physico-chemical Properties--Hexachlorocyclopentadiene (HCCP) has the molecular formula of C_5Cl_6, corresponding to the following structure:



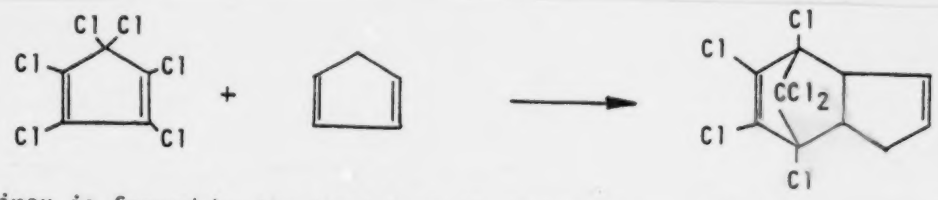
Selected properties of this compound are presented below:

Molecular weight:	285
Melting point:	9.6 ^{o}C
Boiling point:	239 ^{o}C
Specific gravity:	1.717 (at 15 ^{o}C compared to H_2O @ 15 ^{o}C)
Solubility:	Insoluble in water; however, it is soluble in organic solvents such as hexane and methanol.

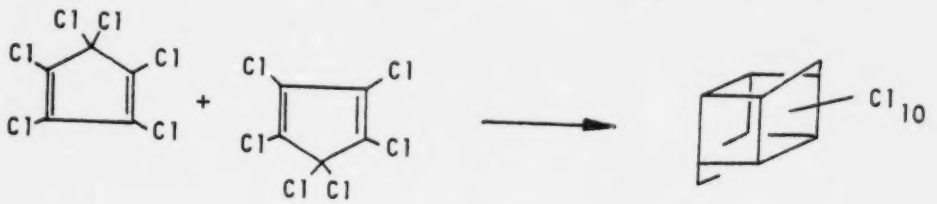
Use and Manufacture--Hexachlorocyclopentadiene (HCCP) is produced in the United States by Velsicol Industries, Inc. and Hooker Chemical Corp. It is manufactured by the reductive dechlorination of cyclopentane. HCCP is used in intermediate cyclo-additions in the preparation of the insecticides aldrin, dieldrin, chlordane, heptachlor, and mirex (Casarett and Doull, 1975). The formation of each of these pesticides is illustrated below.

Aldrin is formed by the Diels-Alder (4+2) addition of HCCP to norbornadiene.

The immediate precursor for chlordane and heptachlor is chlordene, which is manufactured by a Diels-Alder condensation of HCCP and cyclopentadiene.



Mirex is formed by the head-to-tail dimerization of HCCP.



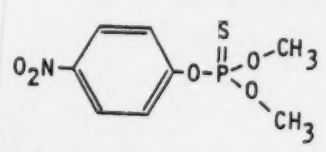
Toxicological Properties--Hexachlorocyclopentadiene is toxic to mammals, including humans, when inhaled, absorbed through the skin, or ingested. The acute oral LD_{50} for rats is 113 mg/kg, and the LD_{50} by skin absorption for rabbits is 430 mg/kg (NIOSH, 1977). Absorption of the compound causes effects in man similar to those resulting from inhalation of carbon tetrachloride vapors (Sax, 1968). A TLV of 10 ppb in air is recommended for the protection of workers (Hawley, 1977).

Environmental Fate--The environmental fate of HCCP has been examined in model ecosystem feeding studies. Although HCCP was quite stable in tested environments, some degradation was observed. No attempt was made to identify the products (Lu, et al., 1975).

No information on the photolytic stability of HCCP was uncovered. Presumably, UV irradiation leads to reductive replacement of chlorine. Replacement initially occurs at the saturated carbon because of the greater stability of the allyl radical compared to the vinyl radical.

Methyl Parathion

Physico-chemical Properties--MP [phosphorothioic acid, O,O-dimethyl O-(4-nitrophenyl) ester] is a white crystalline solid with a molecular formula of $C_8H_{10}NO_5PS$, corresponding to the following structure:



Selected properties of MP are listed below:

Molelcular weight:	263
Melting point:	35-36 ^{o}C
Specific gravity:	1.358 (at 20 ^{o}C compared to H_2O at 4 ^{o}C)
Solubility:	Slightly soluble in water (50 mg/l), soluble in most organic solvents
Decomposes:	120 ^{o}C

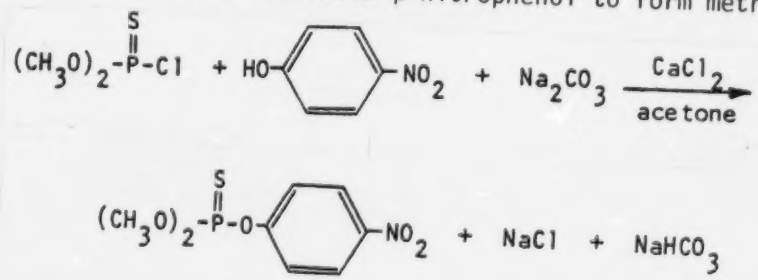
Use and Manufacture--MP is an organophosphate insecticide used in the control of insects on cotton, small grains, and vegetables (Casarett and Doull, 1975). The commercial form is a tan-colored xylene solution. It is manufactured in a three-step process. In the initial step, methanol is added to a suspension of phosphorus pentasulfide.

$$P_2S_5 + 4CH_3OH \longrightarrow 2(CH_3O)_2\text{-}\overset{\overset{S}{\|}}{P}\text{-}SH + H_2S$$

The O,O-dimethyl-dithiophosphoric acid is chlorinated to produce dimethylithiophosphoryl chloride.

$$(CH_3O)_2\text{-}\overset{\overset{S}{\|}}{P}\text{-}SH + Cl_2 \longrightarrow (CH_3O)_2\text{-}\overset{\overset{S}{\|}}{P}\text{-}Cl + HCl + S$$

Then, this chloride is condensed with p-nitrophenol to form methyl parathion.

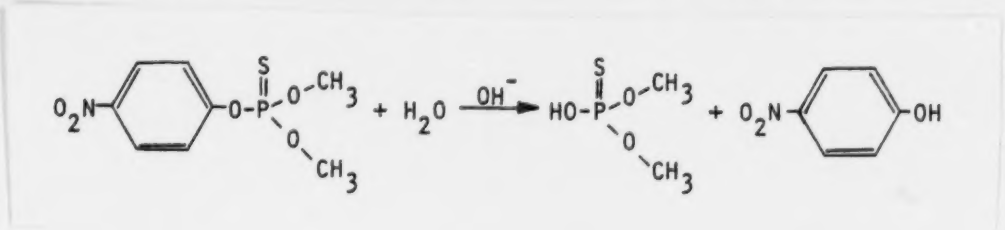


Toxicological Properties--MP is a cholinesterase inhibitor. It is toxic by skin absorption, inhalation, and ingestion. The acute oral toxicity (LD_{50}) of methyl parathion is 14 mg/kg and 24 mg/kg for male and female rats, respectively. Toxicity by the dermal route in both sexes of rats is 67 mg/kg (LD_{50}) (ACGIH, 1977). Studies with humans have shown that plasma and erythrocyte cholinesterase levels remained within 20% of control values when men were fed dosages as great as 9 mg/day for 30 days (ACGIH, 1977). The TLV for MP in air is 0.2 mg/m^3 (ACGIH, 1977).

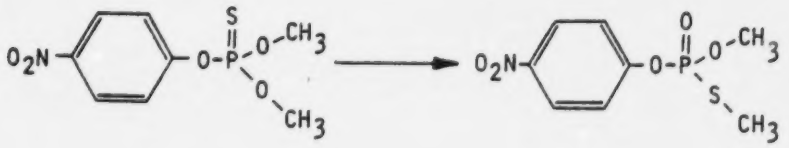
MP in the ppm concentration range is toxic to aquatic organisms. The 96-hour TL_m's for blue gill sunfish and for fathead minnows are 1.9 and 9.3 ppm, respectively (U.S., Coast Guard, 1974).

Environmental Fate-- Methyl parathion is not considered to be a persistent insecticide. It is degradable in the environment by both chemical and biological mechanisms. However, it can persist for several weeks to months depending upon conditions of moisture, concentration, temperature, sunlight, and the availability of organisms capable of metabolizing it.

Methyl parathion hydrolyzes slowly under acidic conditions, but rapidly in a dilute base. During hydrolysis, the nitrophenoxy grouping is replaced (Corbett, 1974).



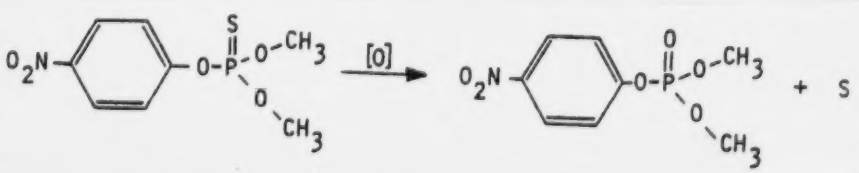
Methyl parathion also undergoes isomerization from a thiono to a thiolo compound.



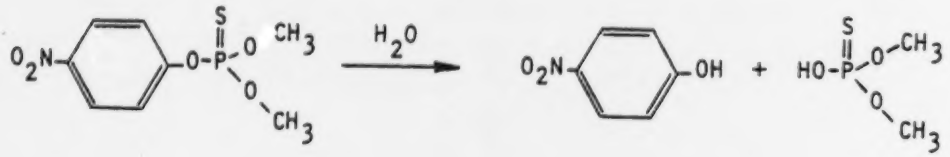
Chemical isomerization is slow at normal temperatures and neutral pH. Higher temperatures and/or increased pH increase the reaction rate. The isomerization product, O,S-dimethyl O-p-nitrophenylphosphorothioate, undergoes hydrolysis more readily than methyl parathion (Corbett, 1974).

Hydrolysis and isomerization may both be catalyzed enzymatically. In addition, several other transformations, illustrated below, occur via biochemical pathways (Casarett and Doull, 1975).

Methyl parathion undergoes a microsomal oxidation at the sulfur atom to form paraoxon (Casarett and Doull, 1975).

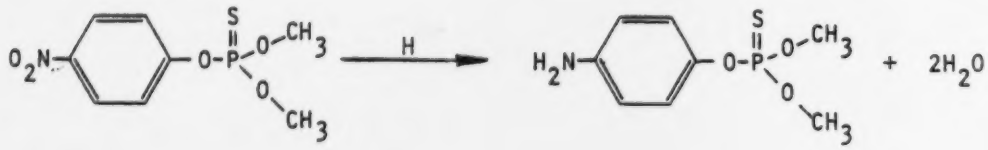


Hydrolysis of MP is achieved in a biochemical detoxification involving the enzymes phosphatase and aryl transferase (Casarett and Doull, 1975).



Biochemical demethylation also occurs (Casarett and Doull, 1975).

Reduction of the nitro group is another biochemical detoxification mechanism and has been observed in polluted waters (Casarett and Doull, 1975).



In addition, the amino derivative has been found as an elimination product from cows fed methyl parathion (Casarett and Doull, 1975; Yasuno, 1965).

SECTION 7

LABORATORY SCREENING EXPERIMENTS

Refer to Section 5 for the experimental details of the screening experiments.

Pentachlorophenol (PCP)

Several PCP screening tests were conducted to select the best organism and to determine the optimum test conditions for degradation of PCP in pilot scale studies. Four candidate organisms showed promise. These were subsequently acclimated to PCP-containing media, transferred, and serially subcultured.

In the first five experiments, aqueous media (pH 6.5 - 6.7) containing basic salts (B.S.), 0.1% dextrose, and various concentrations of PCP were inoculated with different microorganisms. The objective was to provide the highest concentration of PCP at a pH that is optimum for host organisms. PCP is more soluble in alkaline solutions, where it exists in the anionic form, e.g., Na-PCP. Since the solubility of PCP in an aqueous solution at a pH of 6.5 - 6.7 is only about 14 ppm, excess PCP existed as a suspension. Keeping the PCP level high artificially (suspension) did cause some problems in analysis and in biodegradation because of settling out. In some tests, the initial concentration was 700 ppm.

Test mixtures were prepared by adding 2 g of PCP to each liter of medium. This mixture was then heated to 45°C mixed, and filtered. Filtration through a Whatman #3 filter removed undissolved PCP. A second filtration through a 0.45-μ filter was performed for sterilization purposes. One hundred ml portions of the warm filtrate were transferred aseptically into 250-ml Erlenmeyer flasks. Aliquots of the filtrate were assayed for PCP by HPLC.

Screening Test No. 1--Twenty-four organisms grown in ARC No. 50 medium were screened for removal of PCP from the aqueous medium. Each stock culture was centrifuged and 10 ml of cell mass (or cells plus saline) were removed and added to one of 24 flasks containing basic salts and PCP at 700 ppm. Ten ml of sterile saline solution were added to two additional flasks, which served as controls. All 26 flasks were placed on a rotary shaker at 23 °C. After 96 hours, the flasks were harvested by centrifugation. The supernatants and sediments containing the biomass were extracted and analyzed by HPLC.

The results from Experiment 1 are presented in Table 5. These results indicated that fungal isolates 004, 016 and 026 were candidate organisms for removal of PCP from the medium. At this time, the fungus 044 and the

TABLE 5. LABORATORY SCREENING TEST NOS. 1 THROUGH 3: PENTACHLOROPHENOL

Organism	Identity	Test No. 1, Day 4		Test No. 2, Day 4		Test No. 3, Day 6	
		PCP remaining * (ppm)	% Removal**	PCP remaining * (ppm)	% Removal**	PCP remaining * (ppm)	% Removal**
Control	No. 1	685		138		284	
Control	No. 2	700		141		294	
004	Fungus	320	54	118	15	262	9
016	Fungus	362	48	100	28	253	12
026	Fungus	425	39	143	0	272	6

*The control concentration is the initial concentration of PCP and corresponds to 0% removal. The amount remaining includes PCP in solution and in the biomass.

**Percent removal was based on HPLC analysis and was calculated as follows:

$$\left[\frac{\text{Control (avg.)} - \text{PCP remaining}}{\text{Control}}\right] \times 100.$$

bacterium 041 were only regarded as second choice organisms but were subsequently tested in the pilot work. The other 19 organisms were ineffective and not considered further.

Screening Test No. 2--This experiment was designed to evaluate the ability of fungal isolates identified in Test 1 to remove PCP from aqueous medium at concentrations of approximately 100 ppm. The results were disappointing when compared with the data of Test 1. There was uncertainty about the validity of the analyses and, indeed, about the ability of the organisms to biodegrade the PCP.

Screening Test No. 3--This test differed from Test 2 since a larger sample size was used in an attempt to improve the reliability of analytical results. As shown in Table 5, results of Tests 2 and 3 were not consistent with results obtained in Test 1. The reason for this difference is not understood.

Next, acclimation studies were undertaken in which organisms 004, 016, and 026 were serially transferred to media containing 100 ppm of PCP. After four transfers, only one flask showed cloudiness. A loopful for the medium was aseptically cultured on solid nutrient agar and three colonies developed, namely, bacteria 039, 040, and 041. Isolate 039 did not degrade PCP and was not used further.

Screening Test No. 4--The purpose was to evaluate removal of PCP (ca. 300 ppm) by bacteria isolated from contaminted PCP media (isolates 040 and 041).

Screening Test No. 5--In this experiment, there was a re-evaluation of all organisms that appeared to cause disappearance of PCP at high (650 ppm) initial PCP concentrations. As shown in Table 6, PCP disappearance was observed in media from several flasks; however, the results were inconsistent. Inconsistencies appeared to be the result of the high concentration of PCP used (above the solubility limit for PCP at a pH of 6.5 - 6.7) as well as analytical problems.

Screening Test No. 6--In this experiment, an attempt was made to test the repeatability of the 42% reduction in PCP obtained in Test 5 with organism 041. The medium was basic salts plus 0.1% dextrose; the PCP was partly in solution and partly precipitated. The control showed 693 ppm PCP remaining on day 4, organism 041 showed 634 ppm (8.5% removal) and organism 042 was inactive.

Screening Test No. 7--Several changes were made. No dextrose was added to a 100 ppm PCP solution in basic salts medium adjusted to pH 8.5 to effect complete solubilization of the PCP. The analytical technique was switched from HPLC TO GC-EC where the species analyzed for was the acetate derivative of PCP (refer to Section 4). The data are presented in Table 7. The initial concentration of PCP was 100 ppm, but analysis by the acetate derivative method showed only 60 ppm. A decision was made, nonetheless, to continue with the acetate derivative method since replicate measurements were more consistent than they were with HPLC.

TABLE 6. LABORATORY SCREENING TEST NOS. 4 and 5: PENTACHLOROPHENOL

Organism	Identity	Test No. 4, Day 2+ PCP Remaining (ppm)* Total	Sol'n.	Biomass	%-Removal**	Test No. 4, Day 4+ PCP Remaining* Total	Sol'n.	Biomass	%-Removal	Test No. 5, Day 5+ PCP Remaining* Total (ppm) b	%-Removal**
Control	No. 1	291	291	NR		303	303	NR		665	
Control	No. 2	302	297	5		278	268	10		NR	
039		273	273	NR		264	264	NR		a	
040		255	214	41	16	239	206	33	15	a	
041		211	159	52	31	205	135	70	27	a	
004		a				a				598	10
016		a				a				531	20
026		a				a				667	0

+ Basic salts with 0.1% dextrose.

* The control concentration is that measured in the control flask and corresponds to 0% removal. Differences in control concentrations reflect experimental errors.

** Percent removal was based on HPLC analysis and was calculated as follows:

$$\left[\frac{\text{Control (avg.)} - \text{PCP remaining}}{\text{Control}}\right] \times 100.$$

a Designated organism not used in this test.

b Some PCP precipitated by day 5 and is counted as "remaining".

NR Not Reported B bacterium F fungus

Note: In Test 4, Day 4, the %-removal computed only on the PCP remaining in solution (i.e., excluding that in the biomass) follows: 040, 28%; 041, 52%.

In Test No. 7, as well as in Nos. 8 and 9, the medium was filter sterilized and aseptically added to the 250-ml flasks (100 ml medium).

Screening Test No. 8--One-tenth percent dextrose was again added to the medium (it had not been used in Test 7). Organism 044 was recultured after Test 8 and shown to be contaminated with a Gram-negative rod bacterium, designated as 045. The %-removal was better with 041 and 044 (contaminated with 045) than was the case in earlier tests (refer to Table 7).

Screening Test No. 9--Organisms 041 and 044 were compared with the isolate 045. It appears that 045 alone was not very effective (see Table 7).

General Discussion--The data appear to show that 044 (fungus) and 041 (bacterium) should be good choices for pilot-plant runs. Both were evaluated in pilot-scale experiments but testing and analysis were limited by funding constraints (refer to Section 1).

Hexachlorocyclopentadiene (HCCP)

Screening Test No. 1--HCCP is a dense, water-insoluble liquid that is not directly accessible to microorganisms in an aqueous medium. To overcome this problem, it was necessary to either solubilize or suspend the HCCP in a solvent that is miscible with water.

In initial tests, methanol was selected as a solubilizing agent. HCCP was dissolved in methanol, and a known volume of the mixture was added to each of 26 (24 test + 2 control) flasks containing sterile medium (basic salts solution plus 0.1% dextrose). The final HCCP concentration was 1000 ppm. Ten ml of microbial culture were added to each one of the 24 experimental flasks (a different culture to each flask). Sterile saline solution was added to the control flasks. All flasks were placed on a rotary shaker at room temperature. Within a few hours, the HCCP had formed a second liquid phase. The cell mass and the medium from each of the 26 flasks were extracted with hexane after 14 days of incubation. Seven of the flasks exhibited disappearance of HCCP. Analytical results from the seven flasks are presented in Table 8 (Test No. 1). The values in the table represent the total amount of HCCP found in the aqueous solution and in the cell mass. No metabolic products were detected, indicating that HCCP was either assimilated or completely degraded.

Screening Test No. 2--The seven organisms selected from the first screening test were incubated with HCCP in the absence of a solubilizing agent. Each organism was added to a flask containing 30 ul HCCP in 50 ml basic salts solution with 0.1% dextrose. Flasks were analyzed after 4, 7 and 14 days of incubation. The cellular mass and aqueous medium were extracted and analyzed by GC and/or HPLC. Results of the 7 and 14 day analyses are presented in Table 8 (Test No. 2).

Comparison of results from Test 1 (with solubilizing agent) and Test 2 (no solubilizing agent) revealed that 3 of the 7 flasks containing organisms showed greater HCCP disappearance in the presence of the solubilizer. In addition, successive samplings are more readily obtained from a homogenous

TABLE 7. LABORATORY SCREENING TEST NOS. 7 and 8, AND 9: PENTACHLOROPHENOL

Organism	Identity	Test No. 7, Day 7*		Test No. 8, Day 6+		Test No. 9, Day 5+	
		PCP Remaining (ppm)	% Removal**	PCP Remaining (ppm)	% Removal**	PCP Remaining (ppm)	% Removal+
Control		60		49		44	
040	Bacterium	48	20	a	a	a	a
041	Bacterium	55	8	39	21	34	23
042	Bacterium	45	25	a	a	a	a
043	Bacterium	a	a	42	14	a	a
044	Fungus (with 045 as a contaminant)	a	a	30	39	31	30
045	Bacterium	a	a	a	a	39	11

* No dextrose

\+ 0.1% dextrose added.

** Based on GC-EC analysis and calculated as follows: $\left[\frac{\text{Control - PCP remaining}}{\text{Control}}\right] \times 100.$

a Designated organism not used in this test.

TABLE 8. SCREENING TESTS: HEXACHLOROCYCLOPENTADIENE

Organism	Identify	Test No. 1 Day 14[a] HCCP remaining* (ppm)	Test No. 1 Day 14[a] % Removal**	Test No. 2 Day 7[b] HCCP remaining* (ppm)	Test No. 2 Day 7[b] % Removal**	Test No. 2 Day 14[b] HCCP remaining* (ppm)	Test No. 2 Day 14[b] % Removal**	Test No. 3 Day 6[a] HCCP remaining* (ppm)	Test No. 3 Day 6[a] % Removal**
Control	No. 1	635		540		465		850	
Control	No. 1	630		530		425		Not assayed	
006	Bacterium	410	35	320	40	180	60	550	35
016	Fungus	415	34	440	18	250	44	760	11
020	Fungus	410	35	420	21	195	56	760	11
022	Fungus	150	76	410	23	275	38	410	52
QM 9123 (ARC 123)	Fungus	395	38	360	33	235	47	530	38
ATCC No. 1369 (ARC 369)	Bacterium	350	45	450	16	290	35	350	59
ATCC No. 21505 (ARC 505)	Bacterium	265	58	390	27	250	44	770	9

** Percent removal was calculated as follows: $\frac{\text{Control (avg.) - HCCP remaining}}{\text{Control}}$ x 100.

* The control concentration is the initial concentration of HCCP and corresponds to 0% removal. The amount remaining includes that in solution and in the biomass.

(a) HCCP in methanol to make 1000 ppm in medium with 0.1% dextrose added. As methanol was consumed, HCCP appeared in biomass sludge.

(b) 30 μl HCCP in 50 ml medium = 600 ppm. Dextrose 0.1%.

mixture. Thus, additional experiments with solubilizers and emulsifiers were planned.

Screening Test No. 3--This experiment was identical to Test 1 using only the seven most promising organisms (006, 016, 020, 022, 369, 123 and 505). Each organism was added to 2 flasks containing 1000 ppm HCCP medium (1.5 ml of a solution containing 30 ml methanol plus 1.2 ml HCCP added to 50 ml salts solution). Flasks were placed on a rotary shaker at room temperature. On day 6, the entire contents of each flask were extracted and analyzed. The results of this experiment are presented in Table 8, Test 3. Data from these experiments indicate that four of the organisms tested may have potential for biodegradation of HCCP. The organisms identified as candidates for degradation of HCCP include two bacterial cultures (006 and 1369) and two fungal cultures (022 and 9123).

The rate of HCCP disappearance appeared to be relatively slow. In order to improve HCCP accessibility to the microorganisms, several means of emulsifying HCCP were explored.

Emulsification Experiments--Sonication was explored as a method for emulsifying HCCP in aqueous solution. The sonication tests were conducted on a basic salts solution containing 0.1% dextrose and 0.1 ml HCCP. During sonification, the chamber became warm and the odor of HCCP was detected. Analysis of the solution by GC-EC indicated complete loss of HCCP from the mixture.

The following emulsifiers were tested in an aqueous basic salts solution: glycerine, Joy™ liquid detergent, methyl Cellosolve™ (ethylene glycol monomethyl ether), Triton X-305™, and Triton X-100™. Emulsions of 1000 ppm HCCP were prepared and remained stable only when emulsifiers constituted greater than 4% of the total volume. Although some settling occurred, none of the emulsions broke on standing.

Triton X-100 formed the most stable emulsion of the emulsifying agents tested. This material is used in biological systems and was not expected to be toxic or inhibitory to the biotreatment organisms. A method for breaking the emulsion to allow for analysis of HCCP had to be developed. Organic solvents, acidic and basic solutions, and salts were tested, including hexane, triisopropanolamine, methylene chloride, 10 M sodium hydroxide, 12 M hydrochloric acid, sodium chloride, ammonium sulfate, butanol, isopropanol, and tert-amyl alcohol. Tert-amyl alcohol plus a 50% saturated sodium chloride solution appeared to be the best agent for breaking a 7% Triton X-100/HCCP/water emulsion.

The emulsion technique was tested on the HCCP medium inoculated with microorganisms. A number of problems were identified. The rotary action of the shaker onto which the test flasks were placed caused the emulsions to break down considerably. The HCCP settled to the bottom of the flasks and out of contact with the microbes. Because of the presence of cell material in the medium, the Triton X-100 and HCCP emulsions could not effectively be broken and extracted for chemical analysis. Other formulations using various concentrations of Triton X-100, with and without

Cellosolve, were tried. None of these formulations was effective in breaking the emulsion.

Three surface-active agents (Duponol G™, Duponol WAQE™, and Merpol OJS™) were also investigated for their ability to emulsify HCCP. Various concentrations of these three agents were tested with HCCP concentrations of 1000 and 500 ppm. When a solution contained less than 33% emulsifier (relative to HCCP), the emulsion either could not be formed or was broken when placed on a rotary shaker. Merpol OJS appeared to be the best emulsifier of the three. This agent was tested at 35% and 50% of the HCCP solution volume with an HCCP concentration of 500 ppm. During the first 24 hours on the rotary shaker, the 50% solution became clear and the distinctive HCCP smell disappeared. In the same time period, the 35% solution became clear with a distinct odor of HCCP. This solution contained only a few minute HCCP droplets. GC-EC analysis confirmed the loss of HCCP from the medium. Thus, it appears that effective emulsification of HCCP causes its volatilization.

Concluding Remarks--Because of these problems, as well as the impracticality of emulsifying a spilled hazardous material prior to treatment, further experimental efforts on emulsification were abandoned and work on HCCP was halted.

Methyl Parathion (MP)

Seven experiments were conducted to evaluate the ability of selected microorganisms to degrade MP. A summary of the results of these experiments is presented in Table 9. Each of the experiments is discussed below. NOTE: In the table, the first column of "Elapsed Days" corresponds with the first column of "% Disappearance", etc.

Experiment 1--For the first experiment (MP-1), 23 candidate organisms were incubated with approximately 166 ppm MP in basic salts solution with dextrose. The incubation flasks were placed on a rotary shaker in a hood at room temperature.

On the 12th day, samples were removed from each flask for HPLC analysis. The results are presented in Table 9. Since each of the flasks, including the controls, originally contained 166 ppm MP, the unexplained recovery of only 96 ppm in the controls caused concern.

The experiment was terminated before disappearance of MP was complete; the cell mass contained undegraded pesticide. The net amount of MP metabolized was determined by subtracting the quantity found in the cells from the amount that disappeared from solution. In some cases, all of the MP removed from solution was recovered from the biomass. In other cases, the MP was not detected in either the biomass or the aqueous medium.

Experiment 2--Organisms from the seven flasks exhibiting the greatest disappearance of MP compared to the controls in experiment No. 1 were grown on ARC No. 50 medium (MP-2). After 2 days of growth, each culture was concentrated by centrifugation and incubated with 250 ppm MP in basic salts

TABLE 9. BIODEGRADATION EXPERIMENTS WITH METHYL PARATHION (MP)

Experiment	Organism	Elapsed Days	%-Disappearance of MP from Solution Compared to Controls*+	Remarks
MP-1	010	7, 12	63, 0	Basic salts medium plus 0.1% dextrose. Original concentration of MP was 166 ppm. Only 96 ppm recovered in controls.
	016	7, 12	58, 0	
	020	7, 12	55, 0	
	021	7, 12	60, 43	
	022	7, 12	50, 48	
	026	7, 12	45, 27	
	003	7, 12	50, 70	
	123	7, 12	63, 55	
	645	7, 12	53, 60	
	950	7, 12	43, 49	
MP-2	021	6	0	Basic salts medium without added dextrose. Original concentration of MP was 250 ppm.
	022	6	0	
	026	6	5	
	003	6	9	
	123	6	21	
	645	6	0	
	950	6	5	
MP-3	021	7, 10	15, 32	Basic salts medium with 0.1% added dextrose. Original concentration of MP was 250 ppm.
	022	7, 10	8, 0	
	026	7, 10	0, 3	
	003	7, 10	0, 18	
	123	7, 10	45, 28	
	645	7, 10	15, 44	
	950	7, 10	13, 0	
MP-4	003	3, 7, 10	0, 0, 18	Basic salts medium with 0.1% added dextrose. Original concentration of MP was 250 ppm.
	021	3, 7, 10	8, 15, 32	
	026	3, 7, 10	0, 0, 3	
	123	3, 7, 10	18, 45, 28	
	645	3, 7, 10	29, 15, 44	
MP-5	003	4, 11	20, 27	Basic salts medium with 0.2% added dextrose. Original concentration of MP was 250 ppm.
	021	4, 11	11, 3	
	123	4, 11	8, 6	
	645	4, 11	24, 17	
MP-6	003-a	6	42	a = Basic salts medium b = Basic salts + 0.5% dextrose c = Basic salts + 1.0% Bacto-peptone Original concentration of MP was 100 ppm.
	003-b	6	51	
	003-c	6	44	
	021-a	6	37	
	021-b	6	37	
	021-c	6	43	
	023-a	6	32	
	123-b	6	38	
	123-c	6	38	
	645-a	6	43	
	645-b	6	49	
	645-c	6	31	
MP-7	003-b	4	40	b = Basic salts + 0.5% dextrose c = Basic salts + 1.0% Bacto-peptone Original concentration of MP was 100 ppm.
	003-c	4	30	
	021-b	4	45	
	021-c	4	28	

* 0% disappearance or apparent reappearance of MP in samples results from changes in controls which also exhibited disappearance of MP.

+ $\frac{[\text{Initial MP} - (\text{amount in solution} + \text{amount in biomass})]}{\text{Initial MP}} \times 100 = \%\text{-Disappearance}$.

solution without dextrose. The purpose of this experiment was to determine whether dextrose played a significant role in the reduction of MP.

On the 6th day, 5 ml samples were removed from each flask and filtered for HPLC analysis. In the controls, approximately 50% of the MP was recovered. Overall, the percent reduction for all of the test organisms was less than was observed in experiment No. 1.

Experiment 3--The results of experiment No. 1 (with dextrose) were compared with results of experiment No. 2 (without dextrose). The comparison indicated that disappearance of MP was greater in the presence of dextrose; therefore, the organisms used in experiment No. 2 were incubated with MP under conditions described for experiment No. 1. Samples were assayed at 7 and 10 days. After 7 days of incubation, the MP concentration had decreased in five of the flasks (021, 003, 123, 645, and 950). Assay of the sample on day 10 showed a decrease in MP concentration of 44% and 14% for organisms 021 and 003 respectively.

Experiment 4--This experiment duplicated the conditions of experiment No. 3 using five organisms (021, 003, 123, 645, and 026). Samples were assayed for MP at 3 days, 7 days, and 10 days of incubation. Results are presented in Table 9. The inconsistency of results probably resulted from analytical problems and the unexplained reduction of MP in the controls.

Experiment 5--Test conditions for experiment No. 5 were identical to those described for experiment No. 4, except for the dextrose content which was increased to 0.2%. The objective of this experiment was to determine if additional nutrients would enhance the removal of MP. Organism 026 was eliminated as a candidate for methyl parathion degradation based on results of previous tests; thus, four organisms were incubated with MP.

Samples were removed after 4 and 11 days of incubation for analysis of the medium and the biomass. Results of the analysis are presented in Table 9. No evidence that the additional dextrose enhanced the disappearance of MP was observed.

Experiment 6--The objective of this experiment was to compare the ability of selected microbial cultures (021, 003, 123, and 645) to decrease MP concentrations under the following nutrient conditions:

a) basic salts

b) basic salts + 0.5% dextrose

c) basic salts + 1.0% Bacto-peptone.

The selected cultures were incubated with 100 ppm MP in each of the media specified above. Control flasks for each media were also prepared. Samples were taken after 6 days of incubation and analyses were performed by HPLC. Results presented in Table 9 show that organism 003 (bacterium) under Condition "b"), and organism 021 (fungus) under Condition "c") showed the greatest decrease in MP concentration.

Experiment 7--This experiment was performed to check results obtained in experiment No. 6 with organisms 003 and 021 and to determine the effect of adding nutrients during the course of the experiment.

The two experimental media were prepared as follows:

1) 1 liter of basic salts solution received 5 g dextrose (0.5%) [Condition "b"]

2) 1 liter of basic salts solution received 10 g Bacto-peptone (1.0%) [Condition "c"]

3) each liter of medium was aliquoted into 20 flasks (50 ml/flask)

4) flasks were plugged with cotton and autoclaved for 17 minutes at 15 psig for sterilization

5) when cool, each flask received 0.5 ml of a 10,000 ppm methyl parathion-distilled water emulsion. The final methyl parathion concentration was 100 ppm.

One flask of each medium was immediately filtered through glass wool for the time zero control. Remaining flasks were inoculated with microorganisms (4.5 ml of culture in ARC No. 50 medium) and were placed on a rotary shaker in the fume hood.

Samples were removed after 4 days of incubation. Analyses of all samples (filtrates and filters retaining biomass) were performed by HPLC. Results presented in Table 9 show decreases in methyl parathion concentration in flasks containing each of the two organisms under both nutrient conditions specified. Dextrose appears to have an overall advantage over the more costly peptone in promoting the disappearance of MP.

General Discussion--Disappearance of MP varied considerably among experiments. Inconsistent results may be attributed to problems with the analytical procedures. During the experiments, analytical procedures were improved sufficiently so that, beginning with experiment No. 6, consistent results were obtained. Comparison of the data on organisms 003 and 021 in experiment Nos. 6 and 7 illustrates this consistency. The MP work was halted and effort expended on pilot-scale tests with PCP and on tests with PCP-oil leachate.

SECTION 8

PILOT-SCALE TESTS

Pilot Unit Preliminary Tests

Pre-operational dye pattern tests were run on the continuous pilot-scale unit (refer to Section 5). These tests determined mixing and flow patterns within the chambers and allowed for correction of leaks and other problems. To standardize aeration and feed rates, two preliminary runs were made using basic salts solution with 0.2% dextrose and organism 016. The inoculum of organism 016 into each chamber was:

Chamber 1 - 800 ml inoculum

Chamber 2 - 500 ml inoculum

Chamber 3 - 300 ml inoculum

Chamber 4 - 300 ml inoculum.

Organism 016, a fungus, was chosen for these studies because it forms large "puff balls" which are easily detected and followed in culture (refer to Figure 4). Problems encountered during the first test were corrected and a second preliminary run was initiated.

The second preliminary run, with a feed rate of approximately 54 liters (13.5 gallons) per day, operated smoothly. Samples from the feed and all four chambers were removed on the eighth day for BOD (5-day biochemical oxygen demand) analysis to determine the efficiency of the 4-chamber unit. In the work, BOD-5 was regarded as a more significant test than COD; funding did not permit both to be run. The reduction of BOD from chamber to chamber is presented in Table 10.

The survival of organism 016 inoculated as a pure culture into an unsterile situation was monitored during this test. Organism 016 dominated the microbial forms found in all chambers of the unit.

Continuous Pilot-Scale Test No. 1

In the first pilot-scale test, the first three chambers were inoculated with organism 044, a fungus. The system was allowed one day to equilibrate. Because of a foaming problem in the chambers, 3.5 ml of Dow Antifoam B™ were added to the feed on days 2 through 7. The effect of this material on the organism was unknown but not expected to be significant. The liquid feed, pumped continuously into the unit, had an initial PCP content of

TABLE 10. RESULTS OF PILOT-PLANT SHAKEDOWN TESTS

Sample	Dissolved oxygen mg/l	BOD-5 mg/l	BOD-5 Percent reduction (Cumulative)
Feed	1.9	1945	
Chamber 1	0.5	1292	34
Chamber 2	0.6	588	70
Chamber 3	6.2	154	92
Chamber 4	5.45	130	93

approximately 100 ppm at a pH of 8.5. The results of the tests are presented in tabular form in Table 11 and graphically in Figure 6.

More than 90% of the PCP appeared to be removed from the system during the first three days of treatment. The chamber pH values were near 5, and it is possible that PCP was no longer soluble in the medium and had precipitated out of solution. When the pH of the pilot unit remained neutral and above (pH near 8) on day 8, PCP did not disappear from the medium. At pH values of ≥ 7, PCP solubility in aqueous medium is increased. By the eighth day, microbial growth in the pilot unit (organism 044, as well as indigenous bacteria) had decreased significantly. Dextrose was added to the feed mixture on the ninth day and bacterial growth appeared to increase although little revitalization of organism 044 was evident. During the days remaining prior to shutdown, no significant removal of PCP was observed. (Note that there was continuous flow through the pilot plant, but that the PCP-removal decreased markedly after the fifth day of operation.)

Continuous Pilot-Scale Test No. 2

A second pilot-scale test with organism 044 was initiated to study the ability of organism 044 to remove PCP over an extended time period from an aqueous solution containing adequate nutrients and to examine the cause of the pH change and decrease in PCP-removal observed in the first test.

The initial concentration of PCP in the liquid feed was approximately 100 ppm and ranged from 70-100 ppm throughout the experiment. On day seven, a mixture of 4 ml of Dow Antifoam B in 150 ml of water was distributed equally among all chambers to control foaming. Samples for PCP analysis, DO and pH determination were obtained periodically beginning on the third day of operation.

As shown in Figure 7, approximately 80% of the PCP was removed through the fifth day. Thereafter, the removal rate decreased while the pH increased. Most of the total PCP reduction was accomplished in chamber 1.

TABLE 11. RESULTS OF PILOT-SCALE TEST NO. 1

Sample	DO mg/l	pH	Temp. °C	% Removal PCP (Cumulative)
2 days:*				
Feed	9.3	8.7	19	
Chamber 1	8.8	5.2	20	76
Chamber 2	8.7	4.7	19.5	84
Chamber 3	9.5	4.6	20	86
Chamber 4	9.7	4.6	20	95
3 days:*+				
Feed	8.7	8.8	22.5	
Chamber 1	7.8	5.1	22	70
Chamber 2	7.2	4.7	22	79
Chamber 3	8.4	4.7	22	87
Chamber 4	7.8	4.7	22	91
5 days:*				
Feed	5.8	8.3	22.5	
Chamber 1	8.0	6.9	23	27
Chamber 2	8.5	7.2	23	56
Chamber 3	8.6	6.5	23	64
Chamber 4	7.8	6.2	23	75
8 days:‡				
Feed	5.4	8.5	22	
Chamber 1	9.6	8.2	22	0
Chamber 2	9.5	8.2	22	0
Chamber 3	9.5	8.2	22	0
Chamber 4	9.5	8.1	22	0
9 days:**				
Feed	a	a	a	
Chamber 1	5.8	6.5	24	a
Chamber 2	4.0	6.2	24	a
Chamber 3	7.7	6.8	24	a
Chamber 4	8.7	6.8	23	a
10 days:**				
Feed	0.7	8.7	24	
Chamber 1	3.2	6.9	24	8
Chamber 2	6.2	7.0	24	2
Chamber 3	8.8	7.2	24	0
Chamber 4	8.9	7.4	24	0
11 days:**				
Feed	7.0	8.7	22	
Chamber 1	9.3	7.2	22	5
Chamber 2	9.5	7.3	22	10
Chamber 3	9.7	7.3	22	10
Chamber 4	9.7	7.3	22	0
12 days:**				
Feed	1.0	7.9	21	
Chamber 1	2.8	6.8	22	5
Chamber 2	5.1	6.9	22	7
Chamber 3	8.7	7.0	22	12
Chamber 4	9.2	7.2	21	14

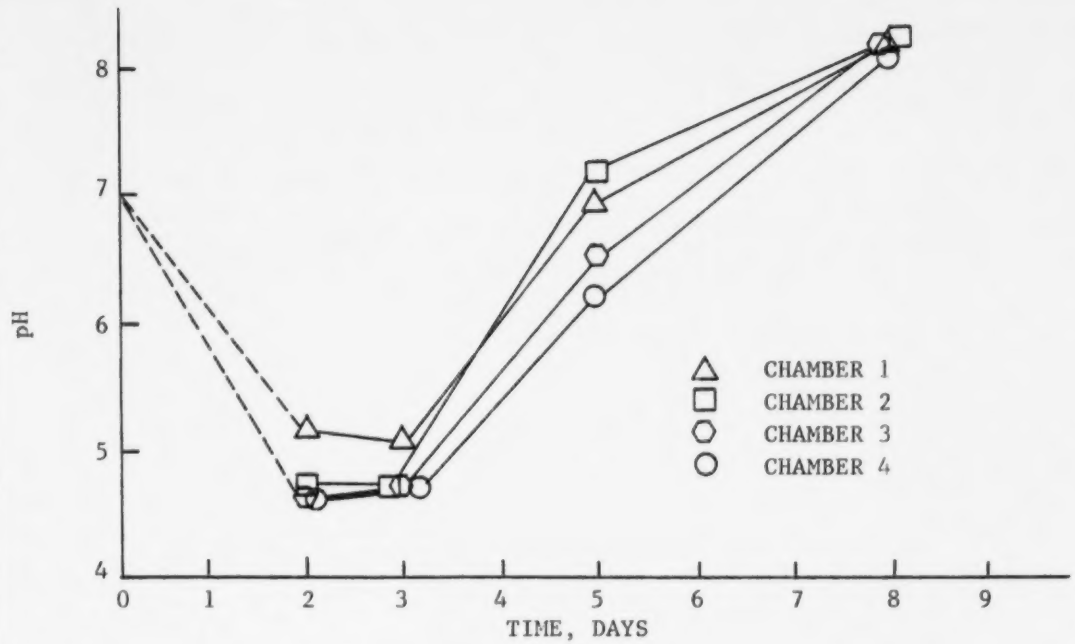
* Dow Antifoam B was added to the feed on days 2 through 7.

\+ Dextrose was removed after sampling on the third day.

‡ Microbial growth decreasing significantly.

** Dextrose was added to the feed mixture.

a No measurement made or calculation performed.



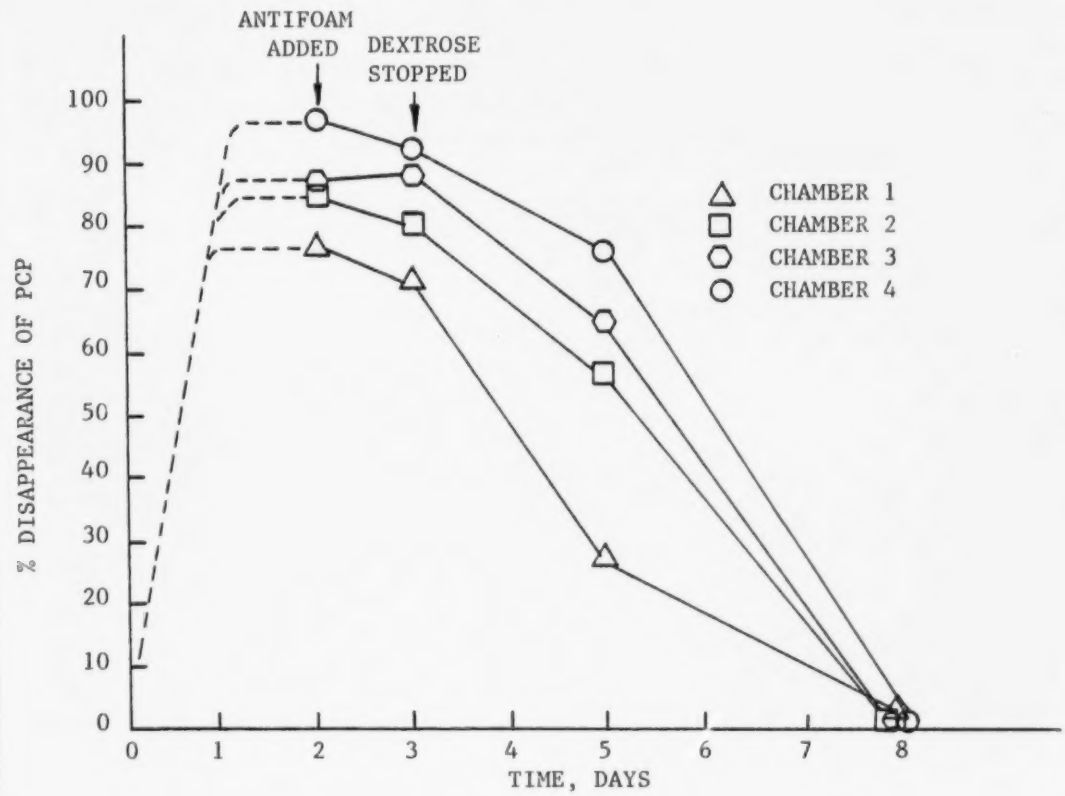
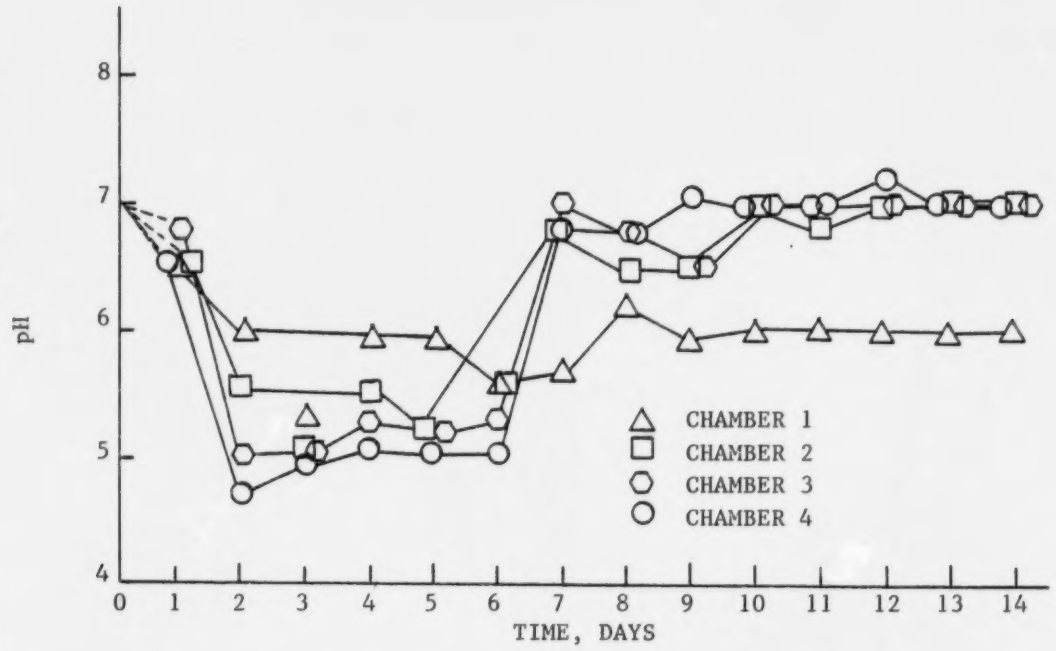


Figure 6. Pilot-scale test No. 1: Pentachlorophenol with fungus 044.



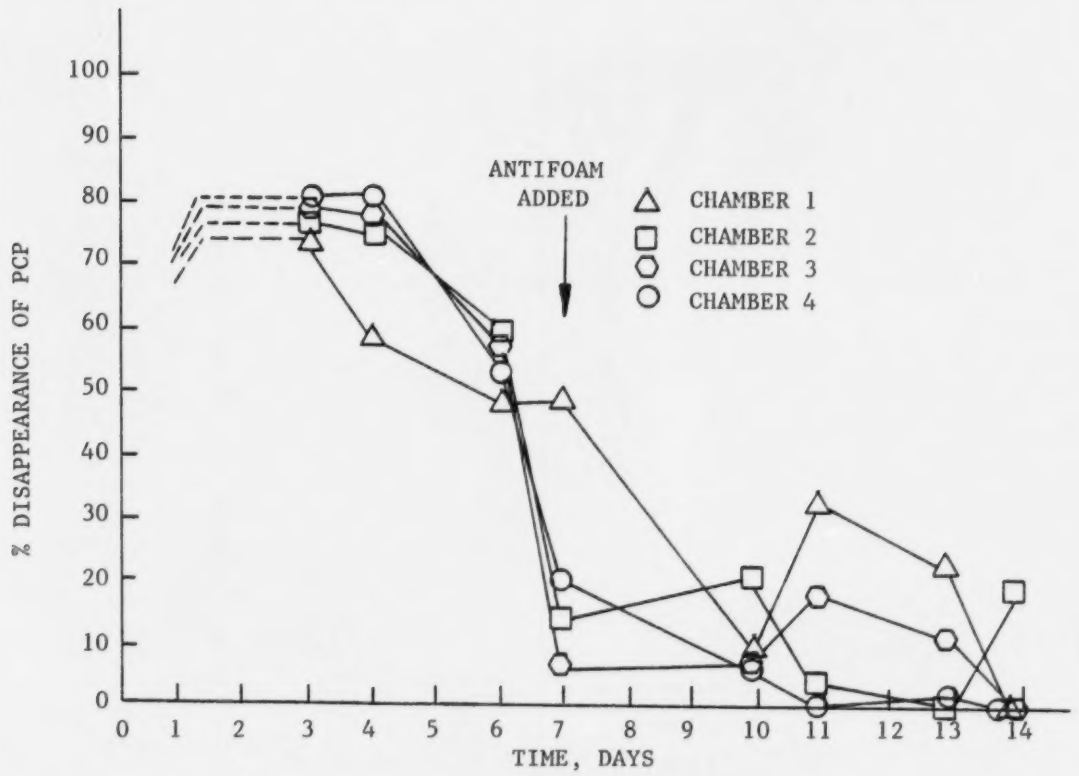


Figure 7. Pilot-scale test No. 2: Pentachlorophenol with fungus 044.

The pH of this chamber remained fairly consistent at ca. 6 throughout the experiment. The amount of PCP removed during the first few days of this test was less than the decrease observed in the first test. This apparent decrease in the amount of PCP disappearance may be the result of the added dextrose which permitted rapid growth of the competing indigenous bacteria. Reduction of fungal growth with a concurrent increase in indigenous bacteria after day 5, indicated that the bacteria were successfully competing with the fungi for available nutrients.

The decrease in pH in the pilot unit was due to acidic metabolites generated by the microorganisms. To determine whether any PCP removal was caused by precipitation due to low pH, bottom samples were collected. One hundred ml samples were withdrawn by suction from the bottom of each chamber. Because biomass constituted 75% of each sample, derivatization of PCP and extraction of the PCP-acetate derivative presented problems. PCP concentrations from the bottom samples were measured in the range of 104 to 525 ppm. pH changes cannot account for the entire decrease in concentration in solution. No definite conclusions can be drawn from these data although they indicate that precipitation of PCP may have occurred and/or that PCP was assimilated by the fungus.

Batch Tests: Studies to Control Bacterial Growth

Sodium azide (NaN_3), a bacteriostatic agent not known to inhibit the growth of fungi, was tested as a means of controlling bacterial growth in the test chambers.

Batch tests were run in 40 liter (10 gallon) glass tanks fitted with constant aeration devices. The tanks were placed uncovered in a hood and filled with 10 liters of water, basic salts solution, dextrose (0.1%), and PCP at a concentration of 100 ppm. The pH of this feed medium was adjusted with NaOH and maintained at 8.0.

The first test was designed to determine the effects of NaN_3 on organism 044. Two tanks were each inoculated with one liter of organism 044. Tank A contained 10.0 g of NaN_3 (final concentration 0.1%) and tank B contained no NaN_3. During a seven-day test period, no bacterial growth or pH fluctuation was observed in tank A. In tank B (control), a substantial bacterial growth and daily reduction in pH values were observed. NaOH additions were required to maintain the pH near 8.0 in the tank.

PCP was added to each tank daily (0.5 g) and additional dextrose and salts were added as necessary. Samples were removed twice daily for PCP-acetate analysis, DO, temperature, and pH determinations.

Results of the GC-EC analysis indicated that there was a few percent removal of PCP in tank B but essentially no PCP removal in tank A in which 0.1% NAN_3 had maintained a low level of bacteria. This level of NAN_3 inhibited Gram-positive bacterial growth strongly. No new fungal growth was observed in tank A and it was concluded that the sodium azide had inhibited organism 044 at the concentration employed in this experiment.

Other batch tests to evaluate the effect of decreased sodium azide concentration showed that a concentration of 0.002% NaN_3 was sufficient to inhibit bacterial growth without having any significant effect on fungal growth.

Continuous Pilot-Scale Test No. 3

This test was initiated to determine whether the addition of 0.002% NaN_3 was feasible in a pilot-scale unit. Organism 044 seemed to grow and reproduce adequately at this concentration and bacterial growth was inhibited. It was not known whether organism 044 was capable of degrading PCP and maintaining active growth in the presence of 0.002% NaN_3 and 100 ppm PCP. In this experiment, the liquid medium remained essentially the same as in tests 1 and 2 except for the addition of 0.002% NaN_3. Assay for PCP-acetate was performed by GC-EC and results of this experiment are presented in Figure 8.

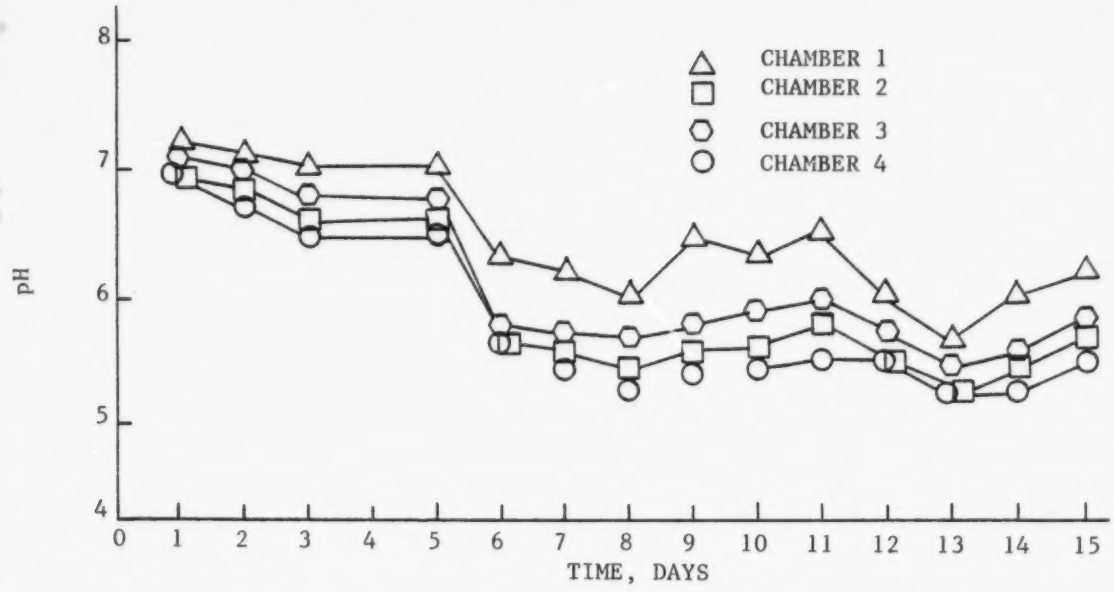
A malfunction in the pilot plant was undetected during a pre-operation check but rendered the unit inoperable after all chambers had been filled with feed and inoculated with organism 044. Because of this malfunction, no air was provided to the organisms during the first two days of operation. PCP removal during the first five days was observed. A new inoculum was added on the fifth day and visual observation revealed that the multiplication of 044 was somewhat inhibited. PCP removal from the medium was observed as the pH of the chambers decreased following re-inoculation with the viable culture. Bacterial growth was suppressed throughout the test but it appeared that organism 044 was stressed by the presence of 0.002% sodium azide.

Continuous Pilot-Scale Test No. 4

This experiment was designed to evaluate the potential of organism 041, a Gram-positive cocci, for degradation of PCP in the pilot unit. The feed medium was altered for this test by replacing NH_4NO_3 with yeast extract (1.0 g/liter). This substitution was made because organism 041 was unable to use the NH_4NO_3 as a nitrogen source. Dextrose was reduced to 1.0 g and NaN_3 was omitted. Daily additions of 3.5 ml Dow Antifoam B were required to control foaming. PCP was dissolved in small amounts of NaOH prior to blending with the feed to yield a 100 ppm concentration.

Visual monitoring of the test organism was not possible; therefore, periodic Gram staining tests were initiated. Dissolved oxygen, temperature, and pH were recorded daily. Samples were withdrawn for PCP analysis, the acetate derivative formed, extracted, and analyzed by GC-EC.

Overwhelming bacterial contamination of the feed reservoir after one day of operation required a readjustment of the feed system. On the third day, the biomass recycle was disconnected from the peristaltic pump and the pilot-scale unit was sealed to prevent loss of the organism. A glass carboy containing 14 liters of sterile solution of water, 0.5% yeast extract, and 0.5% dextrose was attached to the pump. This solution was fed into the system through the port formerly used for biomass recycling. Basic salts



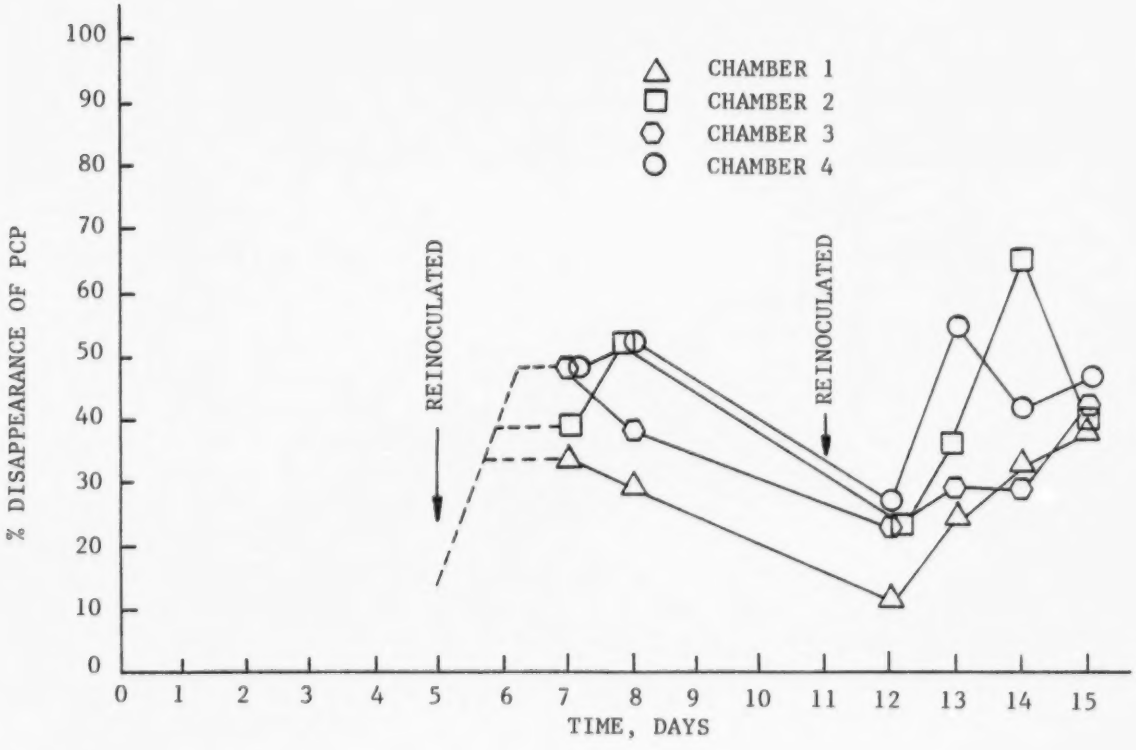
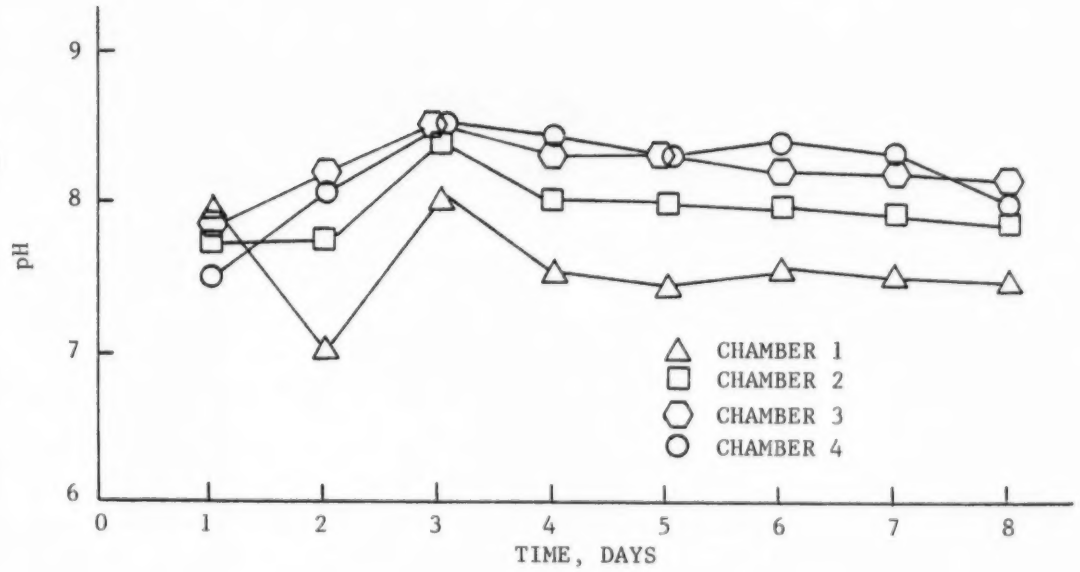


Figure 8. Pilot-scale test No. 3: Pentachlorophenol with fungus 044, 0.002% sodium azide added.

and PCP were supplied to the systems through the established feed reservoir. Effective concentration of both yeast extract and dextrose added to the feed was 0.1%. Altering the feed system eliminated biomass recycle capability which might have promoted the biodegradation process.

Results of this experiment are presented in Figure 9. Variations in the analytical results are probably the result of the large volume of cell mass in the samples, which caused an emulsion in the hexane extract that was incompletely broken. Following shut-down of the pilot unit, organism 041 was found to be the dominant viable organism in all chambers. The pH did not substantially decrease within the chambers. On day eight, the concentrations of yeast extract and dextrose in the feed were decreased to 0.06% in an attempt to force the organism to use greater amounts of PCP for survival. On day ten, chamber turbidity had decreased, indicating loss of active bacteria. No increase in PCP removal was noted and the experiment was terminated on the eleventh day.



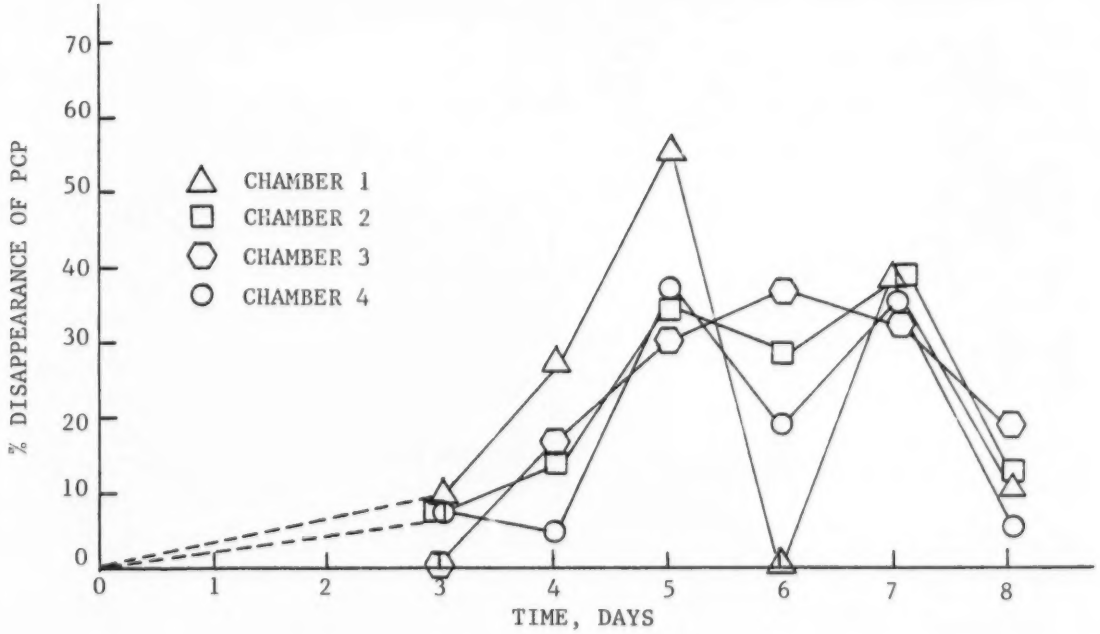


Figure 9. Pilot-scale test No. 4: Pentachlorophenol with bacterium 041.

SECTION 9

PRELIMINARY STUDIES ON A SPILL IN HAVERFORD, PENNSYLVANIA

DESCRIPTION OF THE SPILL AREA

The area surrounding the National Wood Preservers' facility in Haverford, Pennsylvania was contaminated with spilled wood preservative containing pentachlorophenol in No. 2 fuel oil. The waste material was injected into a 7.6m-deep (25 ft.) well located near the plant during the period from 1947 to 1963. The quantity of material injected into the ground was estimated to be between 1 and 3 million gallons (3.8×10^6 to 1.1×10^7 liters). The fuel oil, containing approximately 10,000 ppm PCP, spread into an area 1 x 0.5 miles (1.6 x 0.8 km) and began to slowly leach into Naylor's Run, a small stream located in the area. Since a concentration of 0.2 ppm PCP is lethal to fish, the effects of this discharge were devastating to aquatic life. Additionally, the discharge posed a serious health hazard to humans and wildlife in contact with the contaminated water.

The Environmental Protection Agency (EPA) was notified about the oil-in-water contamination by residents of the area. EPA began a clean-up operation to soak up and dispose of the oil. However, the water soluble portions of the wood preservative, including PCP, were not affected by this treatment procedure. A series of settling tanks was used to separate out the solids for disposal. Then, diatomaceous earth and carbon filters were used to reduce the aqueous PCP concentration to an acceptable level before the treated water was discharged (Lafornara, 1978). Because of the extremely sandy nature of the soil, the wood preservative continued to leach into the water table although the well had been pumped out several times. A series of culture tests were performed by Atlantic Research to examine the microbiological flora of the contaminated area.

SAMPLING

Atlantic Research personnel visited the site. Through the good graces of Mr. Thomas Massey, the On-Scene Coordinator for U.S. EPA Region III, three samples were provided. These samples were obtained from wells within the contaminated area and are identified as follows:

1. near the point of maximum contamination at the National Wood Preservers' (NWP) facility.

2. in the so-called upper discharge area, a location about 200 yards from the first sampling point and near a fence separating NWP from other property.

3. in the lower discharge area, a location about 1.2 km (3/4 mile) from the upper discharge sampling well. This sample at the so-called Washington Avenue area was collected after about 10 min of pumping.

The samples were cultured within four hours of receipt to ensure that reliable data were obtained. All three samples were grown on suitable liquid and solid media in order to support the growth of aerobic and facultative anaerobic microorganisms. Thus, the possible presence of strict anaerobes was not determined. PCP concentrations in these samples were not measured.

Activity of Samples

Sample No. 1 contained less than 2 colony foaming units/ml (2 cfu/ml) and was effectively sterile with regard to bacterial growth. Sample No. 2 contained approximately 8×10^4 cfu/ml. The high bacterial population may have been the result of the presence of oil, a carbon source capable of supporting microbial growth in the soil. The microbial populations found in this sample were different from those normally found in soil. Fungi and actinomycetes, which normally contribute up to 50 - 60% of the microbial population in soil, were not present. The population shift can be attributed to the fungicidal properties of PCP. Sample No. 3 contained approximately 3×10^5 cfu/ml. As in sample No. 2, this number was high and could be attributed to the polluting oil found in the water.

Remarks

No Gram-positive bacteria were found. Although Gram-positives make up a relatively small proportion of normal soils, some should have been present. No members of the Salmonella, Shegella, or Proteus groups (pathogens) were found, as expected. The isolated bacteria all showed Gram-negative staining and were rods. A great proportion appeared to be Azotobacter species as well as possibly Pseudomonas, Acetobacter and Aeromonas. All of these are common soil bacteria. However, there were very few Enterobacteriaceae, such as Escherichia, Enterobacter, and Klebsiella, which make up the great majority of the total coliform group. The samples were not cultured in a manner necessary to detect anaerobic bacteria. Thus, the presence or absence of these type of organisms was not determined.

The bacteria present in sample Nos. 2 and 3 did not appear to be removing PCP. In the 15 years since the end of PCP-oil injection into the ground, very little, if any, "natural" biodegradation of the hazardous material has occurred. The presence of PCP and contaminants, such as chlorodibenzo-p-dioxins, and the possibility of photodegradation of these chemicals into more toxic compounds are of great concern. In order to evaluate the ability of pure culture microorganisms to degrade the PCP-oil mixture in a soil medium, a sample of the PCP-oil mixture was obtained from the U.S. EPA Region III.

WOOD PRESERVATIVE (PCP) SCREENING TESTS

Two types of screening tests were conducted with the spilled hazardous material. In the first tests, an aqueous basic salts medium was employed. Sandy soil, basic salts medium, and dextrose were utilized in the second set of tests.

Aqueous Screening Tests

Some portions of the spilled material, including PCP and the oil impurities, were known to be water soluble. Two experiments were performed to discover whether either the PCP or the oil could be used by microorganisms as a carbon source in a liquid medium. The oil remained essentially unmixed on the surface of the liquid. It was uncertain whether sufficient expose of the organism to the oil could be achieved by a shaker test.

Fungus 044 and bacterium 041 were selected for use in the first experiment based on the results of previous laboratory screening tests. A mixture of ten percent of the Haverford PCP-oil wood preservative sample and 90% of the basic salts solution was used as the test medium. The entire contents of each sample flask and control flask were extracted and analyzed by GC-EC. The analytical results were erratic and no conclusions could be drawn from the data. Because of extreme fouling of the GC column by the residual oil and lack of time and funds, plans for further quantitative analysis were abandoned.

A qualitative screening test with numerous Atlantic Research stock cultures was initiated. Two ml of the PCP-oil mixture were added to flasks containing 100 ml of basic salts solution. The flasks were inoculated with cultures concentrated from liquid media by centrifugation. Two flasks were not inoculated and served as controls. Most of the oil remained unmixed with the medium. However, some exposure to the organisms was achieved by placing the flasks on the shaker rack. Measurements were taken periodically to determine whether the volume or consistency of the oil had changed, indicating metabolic action by the microorganisms. Observations of the flasks over a period of 15 days showed a change in the consistency of the oil. Oil in the control flasks remained smooth while the oil in flasks inoculated with microorganisms showed various degrees of grainy textures. In addition, oil in five flasks was reduced in volume by 1/3 or more when compared to the control flasks. Because of time and funding limitations, no quantitative analysis was performed to precisely measure the volume of oil remaining.

Soil Screening Tests

Sandy soil similar to that found in the spill area was obtained. The concentration of wood preservative/oil in the on-site sand varied; therefore, a series of contaminant concentrations was used to determine optimum conditions for growth of the microorganisms. Degradation of oil or PCP was not quantified during these tests.

Soil Test No. 1--Two organisms, fungus 044 and bacterium 041, were selected for testing under aseptic conditions in order to closely examine the pure culture approach.

Two hundred grams of sand were weighed into each of eight glass beakers which were covered with autoclave paper and sterilized. Pentachlorophenol-contaminated oil (obtained from the spill site) was added to duplicate beakers of sand as follows:

Condition 1 - no PCP/oil (control)
Condition 2 - 4 ml PCB/oil (2% volume-to-weight, equivalent to 200-600 ppm PCP)
Condition 3 - 20 ml PCB/oil (10%, equivalent to 1000-3000 ppm PCP)
Condition 4 - 30 ml PCB/oil (15%, equivalent to 1500-4500 ppm PCP).

The contents of the beakers were mixed well. Four-day cultures of 044 and 041, grown in a liquid medium, were centrifuged and excess medium was removed. Organism 041 (bacterium) was resuspended in 40 ml of sterile basic salts solution plus 0.2% dextrose. Ten ml of this suspension were added to four of the eight PCP/oil-in-sand beakers. Six ml of organism 044 cell mass and ten ml of basic salts solution plus 0.2% dextrose were added to the four remaining soil beakers. All samples were remixed, covered, and incubated at room temperature. Sterile distilled water was added periodically to maintain adequate moisture. Seven days after inoculation, growth was detected visually only in the 044 control. Subcultures were made from each beaker to determine whether any non-visual growth had occurred. Growth was detected in the 041 and 044 controls.

The results of test No. 1 indicate that concentrations of 10% and 15% (volume-to-weight) PCP/oil-in-sand are probably too high to allow degradation due to the toxicity of PCP to the microorganism; concentrations of $\leq$ 2% PCP/oil-in-sand are necessary to allow growth of organism 041 and 044.

Soil Test No. 2--A second experiment was set up to examine lower concentrations of wood preservative in sand. Five beakers, each containing 200 g of soil, were sterilized as in test No. 1. Oil, culture inoculum, and nutrients were added. The following test conditions were employed:

Condition 1 - no PCP/oil (control)
Condition 2 - 1.0% PCP/oil-in-soil (100-300 ppm PCP)
Condition 3 - 0.5% PCP/oil-in-soil (50-150 ppm PCP).

Two beakers were treated as follows: 10 ml sterile B.S. + 0.2% dextrose + organism 041 (conditions 1 and 2); three beakers were treated with 10 ml sterile B.S. + 0.2% dextrose + organism 044 (conditions 1, 2, and 3).

Under conditions 2 and 3, fungal growth (044) was evident four and six days after inoculation, respectively. A visual appraisal could not be made of 041 bacterial growth. On the fifth day subculturing was undertaken. The cultures indicated that the 041 control and condition 2 (1.0% PCP/oil) supported active bacterial growth. Conversely, the fungal subcultures revealed limited 044 growth under conditions 2 and 3. Organism 041 appeared

to be least inhibited by the wood preservative. To confirm these results it was decided to test both organisms with ≤ 2% PCP/oil-in-soil and employ a more thorough subculturing technique.

Soil Test No. 3--Sandy soil was aliquoted in 100g amounts to 10 beakers, covered, and autoclaved. PCP/oil was added to soil beakers as follows:

Condition 1 - no PCP/oil (control)
Condition 2 - 0.25 ml (0.25% volume to weight, 25-75 ppm PCP)
Condition 3 - 0.5 ml (0.5%, 50-150 ppm PCP)
Condition 4 - 1.0 ml (1.0%, 100-200 ppm PCP)
Condition 5 - 2.0 ml (2.0%, 200-600 ppm PCP).

The PCP/oil and soil were mixed thoroughly. Ten ml of organism 041 were added to a set of five soil beakers without prior centrifugation of the liquid medium. This procedure was repeated for the second set of five beakers with organism 044. Ten ml of basic salts solution plus 0.2% dextrose were added to each beaker. The samples were mixed and placed at room temperature in a chemical hood.

After a five-day growth period, the organism 044 (fungus) control beaker was completely overgrown with fungus. Significantly less growth was observed under conditions 2, 3, and 4. No visible signs of fungal growth were seen under condition 5. While there were no visual indications of bacterial growth under any of the conditions, such growth did occur.

Subcultures of all inoculated soil beakers were made on the fifth day using both agar and liquid media. These tests indicated that the growth of fungus 044 was significantly inhibited by the addition of PCP/oil to the soil medium in amounts exceeding 0.5%. Bacterium 041, on the other hand, was more tolerant of PCP/oil in soil with inhibition evident only at the 2.0% PCP/oil level in soil.

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APPENDIX

The purpose of this appendix is to discuss the microorganisms mentioned in the report and some of the basic questions of the biological degradation processes used by the organisms. Most of the organisms used by ARC in the biological testing schemes are fungi, bacteria, and actinomycetes isolated over the years from sources such as soil, water, waste treatment facilities, and other specialized sources. These organisms have been morphologically identified (i.e., their individual and colony structure specified), but, for the most part, have not been identified as to genus and species. Those microorganisms obtained from the American Type Culture Collection are much better identified, of course.

All the microorganisms used are aerobic and thus utilize oxygen in their metabolic systems. They therefore possess the ability to oxidize numerous compounds by activating oxygen through oxygenase systems.

The metabolic pathways utilized by organisms are numerous and very complex. Years of effort can be spent enumerating one metabolic pathway in one organism. Thus, the specific metabolic capabilities of each of the ARC organisms (which number over 60) are mostly unknown. What is known, however, is some of their capabilities in metabolizing certain compounds. These abilites have been determined by previous work on various classes of compounds. Thus, certain organisms can possibly utilize ring compounds, inorganic nitrogen, sulfur-containing side chains, etc. For each specific chemical tested, a number of organisms are selected which, by past experience, are believed to possess the enzyme systems necessary to metabolize that chemical. The organism's ability to remove and degrade a compound is then tested in actual laboratory conditions.

After an organism has been selected and tested for effectiveness against challenging hazardous materials, the next problem that must be addressed is the quantity and identity of the metabolites and by-products produced. (It is possible for these substances to be more hazardous than the original compound.) Even when the actual metabolic pathway is not known, data can be obtained by analytical identification and quantitation of the treated waste components.

When an organism is selected for testing on a specific hazardous material, new questions arise such as the nature and concentration of the chemical growth factors needed to achieve maximal growth, the rates of multiplication of the organisms, and the kinetics of the biodegradation. Once an organism is fully tested and proven to be able to degrade a certain chemical, this organism can easily be made available for field work. It can be grown in large quantities and maintained in a form for use when the need arises, such as following the occurrence of a spill, the appearance of

leachate from a landfill, or the accumulation of liquid wastes in lagoons.

The application of microorganisms to environmental problems is still an art and practical testing is essential. Although the general metabolic pathways of various genus and species of microorganisms are known, specialized pathways utilized with chemicals not normally found in the environment (pollutants such as chlorinated hydrocarbons) are rarely known. Fortunately, since microorganisms multiply and divide so rapidly (20 min to 2 hr on the average), the chance for mutations is extremely high. It is by mutation of some enzyme system that an organism may develop the ability to degrade a selected compound or even a mixture. Indeed, the idea of induced mutation by genetic mutagens is often used to attempt to generate an organism capable of metabolizing a specific toxic or recalcitrant compound.

Thus, it is each particular isolate of an organism which must be tested and its biochemical assets determined. It must be understood, however, that a tailored isolate may not be at all effective in degrading a similar chemical or a mixture of chemicals.

Adaptation ab initio may be necessary in most cases where biodegradation shows promise. The effect of pH, oxygen level, nutrient level, food (dextrose, peptones), etc. may change as degradation proceeds. The sludge from one processing operation may have to be re-processed--even serially--under altered conditions. Note that, even in customary sewage treatment processes, digestion occurs under much different conditions than does secondary treatment.

The field of biodegradation is broad and mostly uncharted. Obviously, work carried out under this modest contract makes barely a dent in the understanding and utilization of biochemical detoxification processes.

Since each organism is so specialized, general review articles are difficult to locate. Two useful, general introductory texts are:

1) The Microbial World
Roger Y. Stanier and Michael Doudoroff
Prentice Hall.

2) The Life of Bacteria
Kenneth V. Thimann
The Macmillan Company

Two good, but rather dated, specific review articles on biodegradation of halogenated pesticides appear in:

1) "Interactions of Halogenated Pesticides and Microorganisms: A Review"
Robert M. Pfister
CRC Press: Critical Review in Microbiology, Vol. 2, 1972.

2) "Biochemical Transormation of Pesticides by Soil Fungi"
J. M. Bollag
CRC Press: Critical Review in Microbiology, Vol 2, 1972.